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# Response of Propionibacterium to acid and low pH: tolerance and inhibition

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Response of *Propionibacterium* to acid and low pH:  
Tolerance and inhibition

by

Jill Louise Rehberger

A dissertation submitted to the graduate faculty  
in partial fulfillment of the requirements for the degree of  
DOCTOR OF PHILOSOPHY

Department: Food Science and Human Nutrition

Major: Food Science and Technology

Major Professor: Bonita A. Glatz

Iowa State University

Ames, Iowa

1996

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## ABSTRACT

Six strains of *Propionibacterium* (three strains of *P. acidipropionici*, one strain of *P. jensenii*, and two strains of *P. thoenii*) were identified as potential acid- and low-pH-tolerant strains from a screen of the culture collection at Iowa State University for acid production and final pH achieved on various carbon sources. The lowest pH at which the strains could initiate growth and remain viable were determined with lactic, propionic, and hydrochloric acid as acidulants. The ability to produce large amounts of acid or achieve low final pH values at the end of culture growth was not directly related to a strain's ability to initiate growth or survive in low-pH conditions.

When grown in batch culture at pH 7, the proton motive force ( $\Delta p$ ) of *P. thoenii* P127 was comprised of only the electrical potential ( $\Delta \Psi$ ); the internal pH remained constant at pH 7. During growth at pH 5, the  $\Delta p$  consisted of both  $\Delta \Psi$  and pH gradient ( $\Delta pH$ ) and the internal pH decreased slowly throughout the fermentation. Growth at low pH was characterized by low specific growth rate, low internal ATP concentrations, and low propionic and acetic acid yields. The overall  $\Delta p$  values were about the same for cultures grown at low or neutral pH, but the individual components of  $\Delta p$  ( $\Delta \Psi$  and  $\Delta pH$ ) were affected by external pH.

Logarithmically growing cells of P127 exposed to mild acid conditions (pH 5.5) for one doubling were more resistant to lethal pH conditions (pH 3.5) than were cells shifted directly from neutral pH to pH 3.5. This acid tolerance response required protein synthesis and allowed the cells to maintain a proton motive force during exposure to pH 3.5. In contrast, the  $\Delta p$  of the unadapted cells collapsed.

Exposure of P127 to acidic pH values caused the cells to manipulate the components of the  $\Delta p$  to maintain a large transmembrane potential. Strain P127 could not initiate growth at pH values below 5.0; in addition, the  $\Delta p$  dramatically decreased when cells were exposed to pH values  $<5.0$ . This may suggest that the cells' inability to maintain a large enough  $\Delta p$  is responsible for growth inhibition.

## GENERAL INTRODUCTION

Propionibacteria are fermentative microorganisms that produce propionic and acetic acids from the metabolism of lactate and/or carbohydrates (21, 75, 90). Strains of the genus *Propionibacterium* are used industrially as starter cultures for Swiss-type cheeses (21), as silage inocula (26, 76), as probiotics (59), and for the industrial production of vitamin B<sub>12</sub> and propionic acid (75).

Propionic acid is an effective preservative for foods and agricultural products (25, 38, 43, 75). It extends the shelf-life of the product by inhibiting molds, bacteria, and yeasts. Currently commercial production of propionic acid is mainly by petrochemical routes. Fermentation is not used industrially because propionibacteria grow slowly and produce relatively low concentrations of acid. In addition, the costs of separation and concentration of the acids make recovery difficult and expensive. If higher yields could be obtained, the economics of fermentation could be improved. Fermentation would allow the use of industrial waste products (whey, corn steep liquor, and beet molasses) as fermentation substrates. Other antimicrobials such as bacteriocins produced by the organisms could increase the spectrum of antimicrobial activity of the culture product (34, 56). The ability to label the fermentation product as "natural" could increase its appeal to some consumers.

A strain of *Propionibacterium* that could tolerate high concentrations of organic acids and/or low pH might produce more propionic acid and be favored

as silage inoculant or probiotic feed additive. In addition, growth at low pH may facilitate downstream processes to remove product acid from fermentation broth.

An examination of the effect of low pH and organic acids on the cell could lead to greater understanding of the mechanism of action of antimicrobials and the process of end-product inhibition by acids during fermentation. The action of organic acids has been attributed both to a generalized inhibition due to low pH and to a more specific inhibition due to action of the anion (37). Some researchers believe that organic acids act as “uncouplers” because the entrance of undissociated acid into the cell followed by its dissociation there allows protons to enter the cell from the external medium (4, 7, 46, 60). The membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane; in this way, the cell becomes deenergized. In contrast, other researchers believe that organic acid toxicity is more closely related to intracellular pH regulation and anion accumulation than to “uncoupling” per se (15, 79, 80, 81, 82).

The specific objectives of this study were to identify strains of *Propionibacterium* that are potential acid- and low-pH-tolerant variants; to determine the pH values that inhibit growth and survival of these strains; to develop the methods to measure the proton motive force of propionibacteria; to measure the proton motive force of *P. thoenii* P127 during growth at neutral and acidic pH values; and to measure the proton motive force of cells of *P. thoenii* P127 during exposure to low pH.

### **Dissertation Organization**

This dissertation follows the alternative format and is divided into three papers. Each paper contains Introduction, Materials and Methods, Results and Discussion, and References sections. Each section is written to conform to the specifications of the journal to which it will be submitted (Journal of Industrial Microbiology and Applied and Environmental Microbiology). Literature review and general conclusion sections are also included. References cited in those sections follow the general conclusion. All experiments, data collection, and data analysis were performed by the candidate.

## LITERATURE REVIEW

### Characteristics and Metabolism

The genus *Propionibacterium* is divided into two principal groups of organisms, the “classical” propionibacteria and the acnes group. This review will concentrate on the classical propionibacteria, in particular their metabolism, industrial importance, and inhibition by the production of propionic and acetic acid and low pH. This group has been referred to as the dairy propionibacteria and consists of the species *P. freudenreichii*, *P. thoenii*, *P. jensenii*, and *P. acidipropionici* (10, 21). These strains have been isolated from soil, silage, raw milk, Swiss cheese, and other dairy products. Some strains are used extensively as dairy starter cultures (39, 49, 75, 90).

The propionibacteria are gram-positive, nonmotile, catalase-positive, nonsporeforming, pleomorphic rods that are anaerobic to aerotolerant (21), and produce propionic acid, acetic acid, carbon dioxide, and vitamin B<sub>12</sub> from the metabolism of sugar and/or lactate (16, 50, 90).

Propionibacteria are metabolically complex organisms that utilize several different substrates: glucose, fructose, sucrose, maltose, erythritol, glycerol, and others (21, 49, 90). They prefer lactate to glucose and L-(+)-lactate to D-(-)-lactate (16, 39, 74, 90). These organisms produce propionate by a cyclic series of reactions through the intermediate succinate. The theoretical yield from 1.5 moles

of glucose or 3 moles of lactate is 2 moles of propionate, 1 mole of acetate and 1 mole of carbon dioxide (16, 32, 90).

The major pathway of glucose metabolism is via the Embden-Meyerhof pathway (90). Lactate enters the pathway in an oxidation reaction that yields pyruvate, which is then converted to oxaloacetate by a transcarboxylation reaction with (S)-methylmalonyl-CoA as CO<sub>2</sub>-donor and biotin as CO<sub>2</sub>-carrier (Figure 1). In addition, pyruvate can also be converted to phosphoenolpyruvate by pyruvate phosphate dikinase. Through the action of phosphoenolpyruvate carboxytransphosphorylase, carbon dioxide is added to phosphoenolpyruvate in a fixation reaction to yield oxalacetate. In the last two reactions, the function of ATP is performed by inorganic pyrophosphate which is used to phosphorylate fructose-6-phosphate to fructose-1,6 phosphate and serine to phosphoserine (32, 90).

Succinate is formed from oxalacetate through the action of malate dehydrogenase, fumarase, and fumarate reductase. Succinyl-CoA is formed in a CoA transferase reaction, rearranged to (R)-methylmalonyl-CoA, and converted to the (S)-enantiomer by a racemase. Transcarboxylation of (S)-methylmalonyl-CoA yields propionyl CoA; propionate is formed by transfer of CoA to succinate (32, 90).

Under aerobic conditions, intermediary metabolism occurs via the citrate cycle; citric acid cycle intermediates have been isolated in propionibacteria (18, 90). Wood (90) stated that the reactions of the citrate pathway may account for variations in the ratios of propionate to acetate that are often found in cultures.

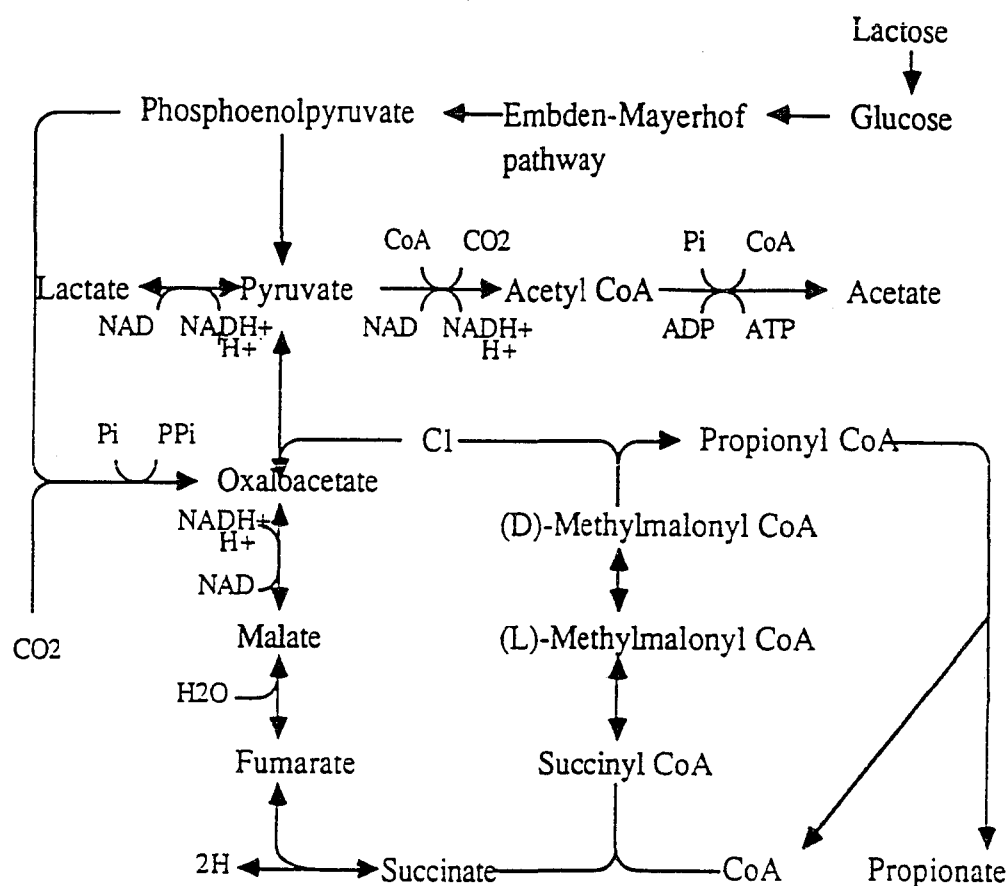


Figure 1: Major metabolic pathway of propionibacteria (adapted from Gottschalk [32]).

In an attempt to determine the effect of pH on these pathways, *P. acidipropionici* was studied in batch culture at pH values controlled at 4.5 to 7.0 with lactose as the substrate (41). The optimum pH for cell growth was 6.0-7.1 (specific growth rate of  $0.23 \text{ h}^{-1}$ ). The growth rate decreased at acidic pH values to a low of  $0.08 \text{ h}^{-1}$  at pH 4.5. Significant amounts of pyruvate and succinate were produced at neutral pH values. However, the pyruvate was reconsumed and disappeared toward the end of the fermentation. Succinate formation



decreased with pH: 17% (w/w) at pH 7.0 to 8% at pH 5.0. Acetic acid yield was not significantly affected by pH. Propionic acid yield (grams of propionic acid produced per gram substrate used) increased from 33% at pH 6.0-7.1 to 63% at pH 4.5-5.0. The authors noted that at acidic pH values, less energy was diverted to succinate and pyruvate formation and thus the propionate yields were higher.

Propionibacteria are capable of utilizing several amino acids, especially aspartate, alanine, serine, and glycine (51). However, there are large differences in such abilities among species and strains. Propionibacteria metabolize aspartate and propionate via the citric acid cycle to yield succinate, ammonia, acetate, and carbon dioxide (16, 18, 77):



Reactions catalyzed by aspartase and carboxytransphosphorylase are also involved. The utilization of aspartate shifts the balance from propionate production to acetate and carbon dioxide formation from lactate, to maintain the redox balance of the cell (16, 18, 74, 77).

Alanine is metabolized after depletion of lactate (16, 19). Degradation of alanine may be delayed by the high concentration of pyruvate in cells from lactate fermentation, which inhibits alanine dehydrogenase. After depletion of pyruvate, alanine is degraded:



Propionibacteria have at least two proteinases, one produced during exponential growth and the other released at stationary phase (24, 51). These enzymes were found in a study of the caseinolytic activity of several strains of propionibacteria. One enzyme, associated with the cell wall, preferentially hydrolyzed B-casein to  $\alpha_{s1}$ -casein. The other had similar activity on both B- and  $\alpha_{s1}$ -casein and was located in the cell membrane. Peptidase activity of several species of propionibacteria has been documented as well (24, 51, 68). The activity varies greatly among species and strains and includes aminopeptidase, proline iminopeptidase, proline imidopeptidase, leucine aminopeptidase, carboxypeptidase and endopeptidases. These enzymes supply the necessary amino-nitrogen for cell growth. In addition, by-products of this metabolism are thought to contribute to flavor in Swiss-type cheeses.

Dupuis *et al.* (23) found *P. freudenreichii* subsp. *freudenreichii* to possess both intracellular and extracellular lipase activity. Lipase activity was detected at the beginning of growth (3 h) and reached a maximum at 7.5 h. The activity quickly decreased after exponential growth. Although other species were found to contain intracellular lipases, no others displayed extracellular lipase activity. The researchers also detected esterase activity in several strains of *P. freudenreichii* subsp. *freudenreichii*. These lipases and esterases are also believed to be involved in flavor development during the ripening of Swiss-type cheese by releasing free fatty acids and other precursors to aromatic compounds.

Crow (20) showed that the propionibacteria produce some polysaccharides during glucose and lactose fermentation. The polysaccharides

contain mostly methylpentoses with some glucose and galactose. He observed that the formation of polysaccharides alters the organic acid ratio by increasing the formation of acetate and carbon dioxide without any associated propionate production.

### Industrial Importance

Many propionibacteria strains are used as starter cultures in the production of Swiss-type cheeses. They are responsible for eye formation and the characteristic sweet and nutty flavors of the cheese (39, 41, 51). In Swiss cheese production, strains of *Lactobacillus* and *Streptococcus* first convert the lactose in milk to lactate, thus providing a substrate for the propionibacteria. In addition, the proteolytic enzymes of these cultures, especially *Lactobacillus*, hydrolyze milk proteins, producing peptides and amino acids that help stimulate the growth of propionibacteria (3, 51, 74). During the curing process, metabolism of lactate yields carbon dioxide, which contributes to eye formation, and propionate and acetate, which contribute to flavor and also extend the shelf life of the product by inhibiting molds, yeasts, and bacteria. The characteristic flavor of Swiss cheese is also due to amino acids, metabolic pathway intermediates, and short-chain fatty acids produced by the propionibacteria (24, 50, 51).

In addition to their use as dairy starter cultures, the propionibacteria can be used to produce valuable products. Propionic acid is an important preservative for foods and grains (25, 26, 38, 43, 75). It exerts a biostatic action in dilute

aqueous systems and is biocidal at higher concentrations. Propionate inhibits growth of molds, bacteria, and yeasts. The undissociated form of the acid is believed to be responsible for its antimicrobial properties. Thus, it is most effective at a pH lower than the  $pK_a$ , 4.87 (25, 38, 43). However, since sodium and calcium salts, which are fully dissociated in solution, also exert an antimicrobial effect, the dissociated form is also believed to be an active species (11, 13).

Buazzi and Marth (11) found that exposure of *Listeria monocytogenes* to a solution of 8% sodium propionate for 60 minutes injured 87% of the population. By studying the recovery of the injured cells in the presence of metabolic inhibitors, they were able to characterize the cellular targets of sodium propionate. Injury did not involve cellular functions related to electron transport, cell wall, cell membrane, mRNA, or protein synthesis. However, there was a reduction of the specific activity of lactate dehydrogenase in the propionate-injured cells. This may be due to propionate inhibiting the synthesis of coenzyme A, which is required for lactate dehydrogenase activity. In addition, the propionate ion itself was the active fraction; 8% NaCl was not inhibitory.

Propionic acid has been found to be more effective than acetate or lactate in inhibiting the acid-tolerant yeasts that cause food spoilage (63). Moon found that some strains of yeast were able to grow in the presence of lactate or acetate but were inhibited by propionate. Even low concentrations of propionate inhibited the transport of amino acids across the cell membrane.

Cherrington *et al.* (14) investigated the use of lactic acid, acetic acid, and BioAdd, a commercial preparation of formic and propionic acids, as potential

decontaminants for scald tank water used in the processing of chicken carcasses. BioAdd was found to have the greatest bactericidal activity against salmonellae. The bactericidal activity increased with temperature and incubation time.

The traditional method of preserving grain is by drying it to 14% moisture (26, 43). However, this process is costly and inefficient because the dried grain must be rewetted before feeding to cattle and sheep. Moist grain has been preserved by spraying with propionic acid, but acid-induced corrosion of equipment is a major disadvantage of this practice.

Raeker *et al.* (76) compared pure propionic acid, sodium propionate, and spent fermentation broth from propionibacteria for their ability to preserve high-moisture corn. Spent fermentation broth was as effective a fungicide as propionate and prevented the growth of *Aspergillus flavus*.

An alternative method for grain preservation is applying propionibacteria as a co-inoculant with lactic acid bacteria (26, 38, 43). Use of propionibacteria and *Lactobacillus* as inoculants for high-moisture corn retarded mold growth and reduced the yeast population over a 60-day period (26). An important advantage of propionic acid treatment over other moist grain storage methods is that its effectiveness is maintained after the grain is removed from storage. In addition, it inhibits the major mycotoxin-producing fungi (28, 38, 76).

There is evidence that propionibacteria have probiotic effects when used alone or together with lactic acid and/or bifidobacteria (2, 12, 59, 88). Probiotics are viable organisms that are nutritionally or therapeutically beneficial to the consumer when ingested in food or feed supplements. The probiotic effect of propionibacteria is based on the production of propionic and acetic acids, carbon

dioxide, bacteriocins, vitamins, as well as their ability to stay alive during digestion.

Propionibacteria have been used in combination with *Lactobacillus acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* and bifidobacteria for successfully curing disorders of the digestive tract of calves, piglets, and hens (2, 88). Cerna *et al.* (12) invented Proma, a blend of *Lactobacillus plantarum*, *Enterococcus faecium*, *Lactococcus lactis* and *P. freudenreichii* cultures. When Proma was fed daily to calves at  $2 \times 10^8$  cfu/g, the calves' daily intake was lower while their daily weight gain was higher than for controls. Even after withdrawal, Proma remained effective; the calves continued to gain weight 11 to 22% faster than the control animals.

Mantere-Alhonen (58) found *P. freudenreichii* subsp. *shermanii* to be an effective probiotic when used alone. Piglets were fed fodder containing *P. freudenreichii* subsp. *shermanii* that had been cultivated in trypsin-treated whey and silage juice. The animals received  $2 \times 10^9$  cfu/g daily for 7 weeks. During that time, the animals gained 9.2 to 14.5% more weight, while their fodder demand was 7.2 to 46.1% lower than for the control animals. In addition, the test piglets experienced less diarrhea than the controls.

Propionibacteria have been investigated as a direct-fed microbial for beef cattle because of their ability to reduce nitrate and nitrite (85). Nitrate from feed is reduced in the rumen to nitrite, which is toxic when absorbed. Nitrite oxidizes ferrous iron in hemoglobin to ferric iron, forming methemoglobin. Nitrate toxicity causes abortions and can be fatal to cattle. A strain of *P. acidipropionici* was found to be capable of reducing high levels of nitrate (15,000 to 20,000 ppm) at

$10^5$  cfu/ml. The culture produced enzymes necessary for nitrate reduction under both aerobic and anaerobic conditions in the absence of nitrate; however, nitrate reductase activity was induced in the presence of nitrate. The induced cultures were able to reduce 80% of medium nitrate in 6 h, while the noninduced cultures took 16 h to reduce the same amount of nitrate. In addition, *P. acidipropionici* was able to establish itself in the rumen at  $>10^5$  cfu/ml. This research led to the production of BovaPro, a direct-fed microbial produced by LaPorte Biochem International, that contains active *P. acidipropionici* cultures.

In addition to their use as animal probiotics, propionibacteria have been studied for their potential benefits to human health. A recent study by Perez-Chaia *et al.* (72) determined that feeding *P. acidipropionici* increased the intestinal level of propionibacteria and affected the lipid metabolism and immune system of mice. When the mice were daily fed  $10^8$  bacteria/ml in nonfat milk for one week, *P. acidipropionici* went from  $10^6$  to  $10^{10}$  bacteria/g in feces and showed an 8-log increase in the small intestine. Reductions of total cholesterol and serum triglyceride concentration were observed for groups of mice receiving skim milk or skim milk with propionibacteria. The HDL levels were also reduced in both groups, but lower LDL levels were found only in mice fed propionibacteria along with milk. Hypolipemic effect was more noticeable in diets with a high lipid content, and was thought to be due to lower intestinal absorption or high lipid metabolism.

At least three products of propionibacteria fermentation are currently sold commercially (92). Microgard, a fermented milk product produced by Wesman Foods, is used to extend the shelf life of cottage cheese. CAPARVE, a food

preservative produced by PTX Food Corp., is marketed as a "natural mold inhibitor." Upgrade, produced by Microlife Techniques, is also used as a preservative. These products are produced by fermentation of natural materials such as milk and whey and are sold as "value-added" products. Food is preserved by addition of the entire fermentation medium.

Currently commercial production of pure propionic acid is entirely by petrochemical routes (75). Fermentation has not been used commercially primarily because propionibacteria grow slowly and produce relatively low concentrations of organic acids, and the costs of separation and concentration of the acids make recovery difficult and expensive.

However, petroleum is an expensive, nonrenewable resource in high demand in today's market, while fermentation substrates are relatively inexpensive, renewable resources. In addition, use of industrial waste products such as whey, corn steep liquor, and beet molasses as fermentation substrates could decrease disposal costs for these materials.

Further advantages of propionate produced via fermentation include the production of bacteriocins, which could increase the spectrum of antimicrobial activity (34, 56) and the ability to label the product as "natural," which increases a product's appeal to some consumers.



## **Acid Inhibition and Tolerance**

A major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid (37, 91). Accumulation of end-products causes a continuous decrease in both specific growth rate and the further production of the product (37). Two ways to overcome this end-product inhibition are to make improvements in the fermentation process to avoid product accumulation, or to develop an acid-tolerant variant of the producer strain.

Fermentation processes that have been developed to achieve greater acid yields include fed-batch, cell immobilization, continuous, continuous cell recycle, semi-continuous, and multi-stage systems. This review will focus on acid inhibition and tolerance; the reader is referred to another review for more information about these processes (75).

## **Bioenergetics**

Acid inhibition of microorganisms has been studied in some detail, but the precise mechanism is still not well understood. The study of bioenergetics provides the background necessary to understand how acid molecules affect cells. Bioenergetics as a discipline is a highly directed search for the solution to a single problem: the mechanism by which energy made available by oxidation of substrates or absorption of light could be coupled to "uphill" reactions, such as the synthesis of ATP or the accumulation of ions across a membrane (35, 67).

The largest advance in bioenergetics was Peter Mitchell's chemiosmotic theory (61, 62), which states that the energized state of a membrane is an

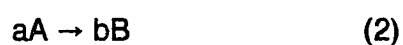
electrochemical proton gradient,  $\Delta\mu_{H^+}$ , that is established by primary proton translocation events that occur during respiration. The  $\Delta\mu_{H^+}$  gives rise to the proton motive force of the membrane.

This theory is derived from equilibrium thermodynamics (35, 67). In a closed system, a process will occur if the entropy (or degree of disorder) of the system plus its surroundings increases. The change in entropy ( $\Delta S$ ) cannot be measured and thus, under constant temperature and pressure, it is calculated from the flow of enthalpy (heat,  $\Delta H$ ) across the boundaries of the system. The relationship is

$$\Delta G = \Delta H - T\Delta S \quad (1)$$

where  $T$  is the absolute temperature (Kelvin). The  $\Delta G$  or Gibb's free energy is the quantitative measure of the net driving force. A process that results in a decrease in free energy proceeds spontaneously (67).

Consider a reaction:



A mass action ratio,  $\Gamma$ , can be calculated:

$$\Gamma = \frac{[B]^b}{[A]^a} \quad (3)$$

At equilibrium,  $\Gamma$  is defined as the equilibrium constant which is denoted as  $K$ .

The  $\Delta G$  is then determined as:

$$\Delta G = -2.3RT \log \frac{K}{\Gamma} \quad (4)$$

where  $R$  is the gas constant (8.3 J/mol K) and  $T$  is the absolute temperature (Kelvin). At equilibrium,  $\Delta G$  is zero and thus there is no energy available for work. The further the reaction is displaced from equilibrium the larger is the capacity of the reaction to do work (67).

This is the case with ATP. Under cellular conditions (pH 7.0,  $[P_i]=10^{-2}$  M) the equilibrium constant for ATP synthesis from ADP and  $P_i$  is large ( $K=10^5$ ) while the mass action ratio is small ( $\Gamma=10^{-5}$ ). This ratio produces a  $\Delta G$  of -57 kJ/mol. This large  $\Delta G$  is the reason why ATP is a source of cellular energy, not because ATP possess a "high energy" phosphate bond. Theoretically, a cell could use any compound as an energy sink as long as its formation reaction was displaced from equilibrium by 10 orders of magnitude or more (67).

These same principles can be applied to the disequilibrium of an ion or metabolite across a membrane. There are two forces acting on an ion gradient across a membrane, one due to the concentration gradient of the ion and one due to the electrical potential difference between the aqueous phases separated by the membrane (membrane potential,  $\Delta \Psi$ ) (35, 67).

Consider the transfer of 1 mole of anion  $X^{m+}$  down an electrical potential of  $\Delta\Psi$  from a concentration  $[X^{m+}]_A$  to a concentration  $[X^{m+}]_B$ . The free energy can be calculated based on the Nernst equation:

$$\Delta G = -mF\Delta\Psi + 2.3RT \log \frac{[X^{m+}]_B}{[X^{m+}]_A} \quad (5)$$

where  $F$  is the Faraday constant (96.519 J/mol mV).

In the specific case of the proton electrochemical gradient:

$$\Delta\mu_{H^+} = -F\Delta\Psi + 2.3RT\Delta pH \quad (6)$$

Mitchell defined the proton motive force as:

$$\Delta p = - \frac{\Delta\mu_{H^+}}{F} \quad (7)$$

$$\Delta p = \Delta\Psi - \frac{2.3RT}{F} \Delta pH \quad (8)$$

The term  $2.3RT/F$  is often designated as  $z$  and has the value of 59 mV at 25°C (35, 61, 62, 67).

This force (interior alkaline and negative with respect to the medium) is generated by primary proton pumps, such as the electron transport chain, which are closely coupled to metabolism (35, 67). This force can then be utilized and

discharged during the function of the proton-translocating ATP synthase,  $H^+$ /solute symporters and other catalysts of bioenergetic work located in the membrane. Typical values of the proton motive force and its components, the electrical potential ( $\Delta\Psi$ ) and the pH gradient ( $\Delta pH$ ), are listed in Table 1 (45).

Fermenting bacteria typically have lower  $\Delta p$  values (<-150 mV) than do respiring organisms (-150 mV to -200 mV). Kashket (46) stated that  $\Delta p$  plays a more limited role in anaerobes which can, under special circumstances, dispense

Table 1: Selected bacterial  $\Delta pH$ ,  $\Delta\Psi$  and  $\Delta p$  values (data from Kashket [45])

Organism	External pH	$Z\Delta pH$ (mV)	$\Delta\Psi$ (mV)	$\Delta p$ (mV)
<u>Respiring acidophiles</u>				
<i>Bacillus acidocaldarius</i>	3.0	230	+10	-220
<i>Thermoplasma ferrooxidans</i>	1.0	340	+70	-270
<u>Respiring neutrophiles</u>				
<i>E. coli</i>	7.5	16	-134	-150
<i>Salmonella typhimurium</i>	7.5	0	-162	-162
<i>Staphylococcus aureus</i>	7.0	32	-162	-194
<u>Fermenting cells</u>				
<i>Streptococcus lactis</i>	5.0	110	-52	-162
	6.0	76	-72	-148
<i>S. typhimurium</i>	7.5		-111	-111
<i>S. aureus</i>	7.0	30	-140	-170
<u>Anaerobes</u>				
<i>Clostridium thermoaceticum</i>	6.0	35	-110	-145
<i>C. thermoaceticum</i> (fermenting)	6.0	71	-95	-166

with  $H^+$  and  $K^+$  circulation (36). This ability was demonstrated by *S. faecalis*, which could grow in the presence of gramicidin D, an ionophore that allows the exchange of monovalent cations across the cell membrane. For the organism to grow, the medium needed to be at neutral pH, low in  $Na^+$ , and rich in  $K^+$ , amino acids and other nutrients. Thus, it appeared that a protonic potential *per se* may not be essential for growth of anaerobes.

Fermenting organisms also differ from aerobes in that they do not maintain a constant internal pH (7, 46, 82). As the bacteria grow they produce acids that lower the external pH. The cells' internal pH decreases with the external pH, although it remains more alkaline than the medium, i.e., the cells maintain a  $\Delta pH$ . The fermenting anaerobes are able to tolerate fairly wide variations in internal proton concentration.

### **Internal pH regulation**

Although fermentative organisms are capable of growth over a wide range of internal pH, it is still important for the cells to regulate their internal pH because the optimum pH ranges of most enzymes are narrow. Regulation of cytoplasmic pH implies control over the cell membrane's permeability to protons. In general, the cell membrane is relatively impermeable to protons. However, protons are involved in almost every biochemical reaction, and thus are produced both outside and inside the cell (7, 69). It is believed that internal pH homeostasis (i.e. maintenance of a constant internal pH) is achieved by control of the activity of ion transport systems that facilitate proton entry.

In a review on pH homeostasis in bacteria, Booth (7) stated several generalizations. First, it appears that all bacteria do not strive to achieve some “magical” internal pH value. It is recognized that neutrophiles have internal pH values in the range of pH 7.5 to 8.0, while acidophiles are in the range of pH 6.5 to 7.0 and alkalophiles are in the range of pH 8.4 to 9.0. Second, organisms exhibit different capacities to regulate their internal pH. Finally, the mechanism of regulation of cytoplasmic pH is not fully understood.

Possible mechanisms for cytoplasmic pH regulation include cytoplasmic buffers and active transport of  $H^+$  (7, 69). The buffering capacity of all cells, regardless of their type of metabolism and pH range of growth, is relatively similar. Amino acid side chains of proteins are the principal components of the buffer capacity. Values of approximately 50 to 100 nmol of  $H^+$  per pH unit per mg of cell protein are typical for the buffering capacity at internal pH 7.0. *Bacillus subtilis* has been found to have an unusually high buffering capacity of up to 200 nmol of  $H^+$  per pH unit per mg protein. However, there is no evidence that a high buffering capacity increases the pH range for growth of the organism (7).

Controlled transport of protons has long been considered to be the dominant mechanism of regulation of cytoplasmic pH. However, Booth (7) stated that the activity of the primary proton pumps is constrained by the generation of the proton motive force. Given a cytoplasmic buffering capacity of 50 nmol of  $H^+$  per pH unit per mg dry weight and assuming  $5 \times 10^8$  cells per mg dry weight, the extrusion of approximately  $4 \times 10^4$  protons, which is enough to generate a  $\Delta p$  of -200 mV, would only raise the internal pH by 0.001 pH units. Thus, the dissipation of excessive membrane potential ( $\Delta \Psi$ ) by either cation influx or anion efflux is

essential for the generation of the pH gradient. For example, the lowering of the internal pH often stimulates  $H^+$ -ATPase activity, but the extent of the stimulation is constrained by the resulting increased membrane potential. An increase in permeability of the cell membrane to ionic species, such as potassium and sodium, is necessary to allow high rates of ATPase to counter the acidification of the cytoplasm. In bacteria, the uptake of potassium, the major cellular cation, during growth may be a major factor in maintaining an internal pH in the range of growth.

### Measurement of the proton motive force

The intracellular pH can be measured by an acid distribution method that is based on the assumption that nondissociated forms of weak acids can diffuse freely through the cell membrane (7, 45, 67, 78). Since the internal and external concentrations equilibrate, distribution of the ionized form becomes a function of the pH gradient across the membrane:

$$\Delta pH = \log \frac{[A^-]_i}{[A^-]_o} \quad (9)$$

where  $[A^-]_i$  is the internal concentration of the anion and  $[A^-]_o$  is the external concentration of the anion of the dissociated acid.

The membrane potential ( $\Delta \Psi$ ) is determined by a similar method. It is based on the uptake of lipophilic cations or anions that distribute across the cell membrane in accordance with the Nernst equation:



$$\Delta\Psi = \frac{RT}{nF} \log \frac{[\text{cation}]_i}{[\text{cation}]_o} \quad (10)$$

Internal volume of the cell is measured from the difference between total and extracellular volumes in cell pellets. The total volume is measured with  $^3\text{H}_2\text{O}$ , and the extracellular volume with large, nonmetabolizable molecules such as inulin, dextrin, or sorbitol. The internal volume is then calculated from the ratio of the radio-labeled large molecules associated with the cell subtracted from the  $^3\text{H}_2\text{O}$  in the cell. The concentration of the probes within the cells can then be calculated (7, 45, 67, 78).

### **“Uncoupler” theory**

Much work has been done using the principles of the chemiosmotic theory to examine the mechanism of acid inhibition and tolerance in microorganisms. One proposed mechanism is that acids may act as solvents that perturb membrane phospholipids. At high concentrations, the acids increase the inward leak of protons so that the proton efflux is not rapid enough to compensate for this leakage and thus the cytoplasm is acidified (46). This action of organic acids had lead to the belief that the acids act as "uncouplers" (4, 37, 46).

The term "uncoupler" was coined by Mitchell in developing the chemiosmotic model of energy coupling (61, 62). Uncouplers are compounds that dissipate the  $\Delta\mu_{\text{H}^+}$  by translocating  $\text{H}^+$  or  $\text{OH}^-$  across the mitochondrial and cell membranes (14, 61, 62). The protonated species travels across the membrane and releases a proton in response to the pH gradient. The anion is

driven to the external surface of the membrane by the electrical potential. The anion is then protonated and the cycle continues. Membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane; thus, the cell becomes deenergized (4, 14, 61, 62).

According to this model, organic acids would act as "uncouplers" that allowed protons to enter the cell from the medium and thus counteract the action of the proton pump (4, 5, 6, 37). The undissociated form of a weak acid can diffuse freely through lipid-containing membranes, and the concentration of the acid inside a cell can become equilibrated with its external concentration (4, 5, 6, 37, 55, 64, 80). However, because the internal pH is higher than the external pH, acid dissociation will proceed to a larger extent inside the cell (Figure 2). As a result, the anion concentration will be higher in the cytoplasm than in the surrounding medium. This results in a net efflux of anions from the cell. The exit of the charged anion is believed to occur when it collides with an undissociated organic acid molecule partitioned in the membrane. This gives rise to a dimer,  $HA_2^-$ , formed by hydrogen bonding within the hydrophobic lipid milieu. Because the species enters as HA and exits as  $HA_2^-$ , for every molecule of acid entering the cell one proton is internalized (4, 37).

There are a number of researchers who credit the action of acids to this "uncoupler" theory. Baronofsky *et al.* (4) studied the effect of acetic acid production during the growth of *Clostridium thermoaceticum*. They followed the internal pH of the cells by measuring the *in vivo*  $\Delta pH$  and  $\Delta \Psi$ . Growing cells maintained a  $\Delta pH$  of about 0.6 throughout most of batch growth, while the  $\Delta \Psi$  decreased from -140 mV at the beginning of growth when the medium was at pH

7.0 to -80 mV at the end when the pH had declined to 5.0. Corresponding  $\Delta p$  values were -155 mV at pH 7 and -120 mV at pH 5. Below pH 5, the  $\Delta p$ H and  $\Delta \Psi$  collapsed, which suggests that the cells required an internal pH of at least 5.5 to 5.7. The authors stated that the low internal pH inhibited pH-sensitive cellular reactions, which lead to cessation of growth and fermentation.

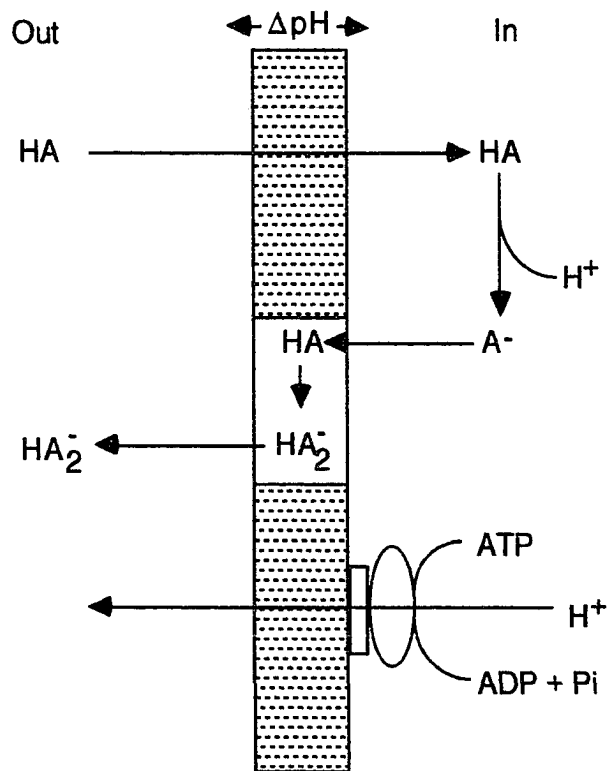


Figure 2: Schematic representation of organic acid movement in bacteria in response to the pH gradient (Adapted from Russell [82]).

When nongrowing cells of *C. thermoaceticum* were resuspended in citrate phosphate buffer at a wide range of pH values, the cells were able to maintain a significantly higher  $\Delta\text{pH}$  and  $\Delta\Psi$  than was observed for growing cells (4). The addition of acetic acid caused the  $\Delta\text{pH}$  to dissipate and the  $\Delta\Psi$  to decrease. Baronofsky *et al.* proposed that organic acid fermentation end-products acted as uncouplers and short-circuited the  $\Delta\text{pH}$ . Furthermore, the proton motive force of the cell collapsed when metabolism could not supply the ATP required for proton extrusion. This proposed mechanism is based on the assumption that bacteria possess a minimum internal pH for growth and that the cell will strive to maintain this pH by extruding  $\text{H}^+$  by means of the membrane-associated  $\text{H}^+$ -ATPase.

Gatje and Gottschalk (30) examined the effect of increasing undissociated lactic acid and proton concentrations on *Lactobacillus helveticus* in continuous and batch cultures. In continuous culture without pH control, the final pH of the medium was 3.8, with 65 mM undissociated lactic acid present. Batch culture experiments showed growth limitation occurred at 60-70 mM lactic acid. After growth ceased, production of lactic acid continued until a concentration of 100 mM was reached. Thus, an uncoupling of growth and acid production occurred. The authors suggested that the energy gained by lactate production was no longer available for cell growth but it was used for the maintenance of  $\Delta\text{pH}$ .

To determine if the undissociated acid or proton concentration caused the uncoupling, *L. helveticus* was grown in batch culture over a range of pH values with lactic or hydrochloric acid as acidulant (30). At similar pH values the culture behaved similarly in the presence of the different acids. The amount of undissociated lactic acid was considered to be primarily responsible for inhibition

of growth and lactic acid production, since this acid was more inhibitory than hydrochloric acid. The researchers concluded that inhibition was due not only to the acidification of the cytoplasm, but also to other physiological effects of the acid produced.

Low intracellular pH lead to inhibition of growth and metabolic activities in other strains of lactic acid bacteria as well. Nannen and Hutkins (65) measured the  $\Delta\text{pH}$  of several lactic acid bacteria during growth. They found that when *Streptococcus thermophilus* was grown without pH control in three different media the cells maintained a  $\Delta\text{pH}$  of approximately 0.8 to 1.0 pH unit during log phase (medium pH declined from 6.5 to 5.5), but this decreased to 0.5 unit after 10 h of growth (medium pH was 4.5), regardless of the buffering capacity of the medium. Continued incubation for 24 h lead to a collapse of  $\Delta\text{pH}$ .

*Lactococcus* species also maintained large  $\Delta\text{pH}$  values during batch growth: 0.97 to 1.40 for *L. lactis* subsp. *lactis* C2, and 0.81 to 1.39 for *L. lactis* subsp. *cremoris* HP (65). However, *L. lactis* subsp. *cremoris* HP experienced a rapid decline in  $\Delta\text{pH}$  after about 6 to 8 h of growth (medium pH was 4.6 to 5.0), while *L. lactis* subsp. *lactis* C2 maintained a  $\Delta\text{pH}$  of approximately 0.6 units for up to 72 h. *Lactobacillus casei*, which reportedly grows at the lowest pH values of lactic acid bacteria, maintained an internal pH near 6.0 when the external pH was  $<4.5$  and a large  $\Delta\text{pH}$  ( $>1.2$ ) when the external pH was  $<4.0$ . This may explain why *L. casei* grows at pH values lower than the streptococci. The researchers concluded that since lactose was still present at the end of fermentation for all lactic acid bacteria studied, cessation of growth was due to dissipation of  $\Delta\text{pH}$ , not

carbohydrate depletion. Growth and metabolic activities were inhibited as a consequence of the deleterious effects of a low intracellular pH.

In a companion study, the  $H^+$ -ATPase activity of these lactic acid bacteria was measured (66). The pH optima for the  $H^+$ -ATPase enzymes of *S. thermophilus* and *L. lactis* subsp. *cremoris* HP were 7.5, while that of *L. casei* was 5.0. For all species, the  $H^+$ -ATPase activity was always greatest when the cytoplasmic pH was less than the optimum for the enzyme. The  $H^+$ -ATPase activity generally increased as the external pH decreased until pH 5.0 was reached. Below pH 5.0, the enzyme activities decreased markedly. Among these organisms, *L. casei* had the highest basal level of  $H^+$ -ATPase. The authors suggested that the  $H^+$ -ATPase enzyme may be involved in the regulation of the intracellular pH in lactic acid bacteria.

Other researchers have investigated the pH optima of enzymes of *L. casei*, with similar results. Bender and Marquis (6) measured the optimal glycolytic pH and membrane ATPase activity of *Lactobacillus casei* and *Actinomyces viscosus*, two organisms found in dental plaque. The authors found that *L. casei*, the acid-tolerant organism, had an optimal glycolytic pH of 6.0 and that a 50% reduction in glucose degradation occurred at pH 4.2. For *A. viscosus*, the acid-sensitive organism, the corresponding pH values were 7.0 and 5.6.

The optimal pH for the ATPase of *L. casei* was approximately 5.0; that for the *A. viscosus* enzyme was pH 7.0 (6). In addition, in the presence of dicyclohexylcarbodiimide, a specific ATPase inhibitor, the proton permeabilities of both organisms markedly increased. Bender and Marquis suggested that ATPases played a role in moving influxing protons back out of the cell.

The authors concluded that acid tolerance could be attributed to greater resistance of the membrane and cellular enzymes to acid damage, higher specific activity of ATPase in the cell membrane, lower pH optimum of ATPase, and greater capacity to expel protons at lower pH (6, 84).

Lactic acid bacteria have been found to increase their ATPase activity in the presence of propionate as well. Perez-Chaia *et al.* (70, 71, 72) have investigated the effects of propionic acid on lactobacilli. In the presence of a low concentration of propionate (0.7 g/L), *Lactobacillus helveticus* had a higher growth rate and biomass yield than the control culture. When the concentration of propionate was higher (>1.4 g/L), the growth rate and biomass were reduced, but the fermentative activity was increased, i.e. cells consumed more glucose and produced more lactic acid than did the control cultures. Increasing the glucose concentration counteracted these effects. Glucose provided the cells with more metabolic energy to counteract the inhibition of propionate. In contrast, *L. casei* was not inhibited by propionate regardless of the glucose concentration.

The membrane-bound ATPase activity of cultures grown in the presence of propionate was higher than for control cultures (70). The pH optima and  $K_m$  values for ATP were identical for the increased and basal ATPase activities for both organisms. Thus, it appeared that the same enzyme was amplified, rather than that a new, more active enzyme was induced. Substrate consumption of resting cells was increased by propionate. In the presence of an ATPase inhibitor, dicyclohexylcarbodiimide, this effect reverted, which indicated that the increase of fermentative activity was related to the  $H^+$ -ATPase activity.

By increasing the fermentative activity, the cells were able to extrude protons from the cytoplasm by means of the  $H^+$ -ATPase. This enzyme utilizes glycolytically generated ATP to expel protons accumulated by the cell as a result of lactic acid production and exposure to propionate. Growth rate and biomass production decreased when metabolism could not supply ATP required for proton extrusion. The tolerance of *L. casei* to propionate may be a result of its high basal level of  $H^+$ -ATPase (66).

In a review of the bioenergetics of lactic acid bacteria, Kashket (46) stated that the minimum pH values for growth and fermentation differ among the lactic acid bacteria probably because of the slightly different complement of their enzymes and transport carriers. Cessation of growth and fermentation occur when the internal pH reaches a given value, which is characteristic of the organism. Internal enzyme activity can only tolerate a pH above a critical value, and this threshold pH depends on the organism. It is not clear whether these cells regulate the internal pH through systems that respond to perturbations in internal pH by restoring the pH to the original value, or whether the internal pH results from all the  $H^+$ -translocating activities taking place at the time without being under the control of a specific pH-sensing regulator.

In the presence of large concentrations of fermentation end-products, the cells eventually are no longer able to expel protons rapidly enough to keep the cytoplasm alkaline with respect to the medium (46). The uncoupling effect of permeant organic acids and the chaotropic effect of solvents are responsible for the increased proton leakage into the cell.



Similar effects of organic acids have been found in other bacteria as well. Luli and Strohl (55) correlated the accumulation of acetate with reduced production of recombinant protein during recombinant *E. coli* fermentations. The toxic effects of acetate were demonstrated at pH values at or below the  $pK_a$  and even at neutral pH when sufficient amounts of acetate had accumulated in the medium. The authors noted that homeostatic mechanisms (mechanisms to maintain a constant internal pH) of *E. coli* required energy to adjust to a decrease in intracellular pH. If not enough energy was available the proton motive force collapsed and growth and fermentation ceased.

### **Anion accumulation theory**

Other researchers question the idea that organic acids act as “uncouplers.” Although the undissociated form of the acid is able to diffuse through the membrane, the anion cannot. Thus, the acid would not continue the futile cycle of transporting protons across the membrane. Russell (82) states “the expansion of ‘uncoupling’ to include any type of metabolic inhibition would create a term with little biochemical meaning.”

Russell (82) suggests that the intracellular release of protons by organic acids is overemphasized. For example, consider a 1 M increase in intracellular acid concentration for a bacterium in which the  $H^+$ /ATP stoichiometry of the membrane-bound ATPase is 3:1 and the intracellular volume is 3  $\mu$ l/mg protein. Based on these assumptions, 1.0  $\mu$ mol ATP/mg protein would be required to expel the protons brought in by the acid. Even anaerobic bacteria with moderate growth rates can produce more than 50  $\mu$ mol ATP/mg protein.

It was observed that some bacteria allowed their internal pH to drop as the extracellular pH decreased (80). Cells of *Streptococcus bovis* grown in the presence of 100 mM acetate allowed their internal pH to decrease to 5.4 before growth was inhibited. Acetate distributed across the membrane in accordance with the Henderson-Hasselbalch equation (on which the measurement of  $\Delta\text{pH}$  is based). Because the  $\Delta\text{pH}$  remained nearly constant, the acetate anion gradient across the membrane remained the same (approximately 10-fold) regardless of the external pH. If *S. bovis* had maintained a constant internal pH, the acetate gradient would have been almost 1500-fold, resulting in an internal concentration of greater than 5 M. Thus, the bacterium's ability to tolerate increasingly acidic conditions was linked to its ability to decrease its internal pH.

When *Streptococcus bovis* and *Lactococcus lactis* were grown with an excess of glucose, lactic acid accumulation caused a decrease in extracellular pH (15). The *S. bovis* culture grew in medium at pH values as low as 4.9, but *L. lactis* was unable to grow below pH 5.3. Both bacteria decreased their internal pH as a function of external pH; the  $\Delta\text{pH}$  was relatively constant. Neither culture showed a significant decline in  $\Delta\psi$  until the medium was below pH 4.8. The glycolytic rate of *S. bovis* decreased as the intracellular pH decreased, but intracellular ATP levels did not decrease. Thus, it appeared that metabolic energy was not the critical aspect of pH sensitivity. Cook and Russell (15) postulated that biosynthesis was pH-sensitive. However, it appeared that metabolic energy was a key factor in the pH-sensitivity of *L. lactis*; the glycolytic rate and the intracellular ATP concentration decreased as the medium pH declined.

Russell (79, 80, 81, 82) concluded that the critical aspect of a particular bacterium's pH sensitivity may vary. However, fermenting bacteria must decrease their internal pH and maintain a low  $\Delta\text{pH}$ . A high  $\Delta\text{pH}$  would cause a large and toxic accumulation of fermentation acid anions. He suggested that the organic acid toxicity was more closely related with intracellular pH regulation and anion accumulation than with "uncoupling" *per se*.

Other researchers agree that acid inhibition is due to anion accumulation. Brink and Konings (9) found that the internal pH of *Streptococcus cremoris*, when grown in batch culture without pH control, remained constant over only a short period and then decreased gradually with external pH. Directly after inoculation no  $\Delta\text{pH}$  could be detected; the  $\Delta\text{pH}$  gradually formed during culture growth and acidification of the medium. The maximal value of  $\Delta\text{pH}$  reached -35 mV when the external pH was 5.7, while  $\Delta\psi$  remained nearly constant (-85 mV to -90 mV) during growth. Total  $\Delta\text{p}$  increased from -90 mV to -122 mV. At the end of growth both components dissipated completely. Internal lactate concentration decreased during growth, while external concentration increased, which corresponded to the lowering of the internal and external pH values. When growth stopped, the internal and external lactate concentrations equilibrated. Growth proceeded for about 6 h and then stopped abruptly after all carbon source was depleted. Even though the external pH decreased from 6.8 to 5.3, the acidification of the medium did not stop growth; when lactose was added growth resumed.

In a study examining the intracellular pH response of *Leuconostoc mesenteroides* and *Lactobacillus plantarum* to external pH changes created by acid production or by addition of lactic or acetic acids to the medium, McDonald et

*al.* (60) found that the internal pH of both bacteria declined with exposure to acidic pH. Growth of *L. mesenteroides* stopped when its internal pH reached 5.4 to 5.7 while growth of *L. plantarum* stopped at an internal pH of 4.6 to 4.8. Variation in growth medium composition or pH did not alter the growth-limiting internal pH. The *L. plantarum* was more acid-tolerant and was able to maintain a  $\Delta\text{pH}$  even when external pH reached 3.0 with 160 mM sodium acetate or lactate. In contrast, the  $\Delta\text{pH}$  of *L. mesenteroides* was zero at pH 4.0 in the presence of acetate and at pH 5.0 in the presence of lactate. The low growth-limiting internal pH and ability to maintain a pH gradient at high organic acid concentration contributed to *L. plantarum*'s acid tolerance.

These researchers stated that lactic acid might affect pH homeostasis through a mechanism which is not solely dependent upon the undissociated acid molecule or  $\text{H}^+$  concentration (60). At all pH values studied, lactic acid was more effective than acetic acid at decreasing the internal pH of *L. mesenteroides* even though the undissociated concentration of acetic acid (136 mM) was higher than that of lactic acid (9 mM). Thus, the authors suggested that lactic acid inhibition was caused by the anion attacking a specific target and not just by acidification of the cytoplasm.

Other researchers agree that organic acid inhibition is due to more than just low internal pH. Salmond *et al.* (83) investigated the effects of organic acids on the growth and intracellular pH of *E. coli*. They grew *E. coli* in the presence of cinnamic, propionic, benzoic or sorbic acids. At higher external pH values (>5.0), a high concentration of acid was necessary to cause complete inhibition of the culture. For all four acids, growth was not inhibited until the internal pH was

below 7.1. Severe inhibition occurred once the internal pH dropped below 6.8. In an effort to determine which form of the acid (undissociated or dissociated) caused the inhibition, the effect of the ratio of benzoic acid and benzoate anion was studied. Different ratios were achieved by varying the undissociated acid concentration at a constant pH or by changing the pH of the medium at a constant acid concentration. Growth inhibition strongly correlated with the concentration of undissociated acid, regardless of how it was achieved.

Salmond *et al.* (83) determined that the acids did not exert their effects simply as a function of their excessive accumulation which would cause acidification of the cytoplasm. If this were the case, the pattern of inhibition would reflect the pK<sub>a</sub> of the acids. Despite the fact that these acids have similar pK<sub>a</sub> values (propionic acid 4.87, sorbic acid 4.77, benzoic acid 4.20, and cinnamic acid 4.44 [22]), they displayed significant differences in their effectiveness as growth inhibitors. Cinnamic acid was found to be the most inhibitory followed by benzoic, sorbic, and propionic acids. It was observed that the predicted intracellular concentration of the dissociated weak acids did not correlate with either the pK or the severity of growth inhibition. The authors concluded that growth inhibition by organic acids had two components: a specific inhibition due to an unidentified metabolic function by the undissociated acid, and a generalized inhibition by the acidification of the cytoplasm.

### **Shift in fermentation pattern**

Fermentative organisms obtain less energy from substrates than do respiring organisms; thus, many fermentative organisms adapt to low pH through

mechanisms that are not energy-consuming, such as shifting their fermentation pattern to producing neutral products such as butanol, ethanol, and acetoin. Tseng *et al.* (87) studied the effects of a shift from pH 5.5 to 7.5 on a steady-state *Lactobacillus plantarum* anaerobic chemostat culture. The  $\Delta p$  decreased 50% (from -106 mV to -55 mV) as a result of the depletion of the transmembrane proton gradient. The internal pH rose from 6.6 to 7.3, while the  $\Delta \Psi$  partially compensated for the lowering of the  $\Delta pH$  by increasing from -42 mV to -63 mV. Intracellular ATP displayed an immediate 20% increase, but then decreased quickly. The authors suggested that the transient increase may have been due to *L. plantarum* shifting to producing some acetate at alkaline pH, which generated an additional ATP by acetate kinase. The overall decrease in ATP concentration may have been caused by the export of protons or ATP used to drive the uptake of nutrients. The  $H^+$ /lactate stoichiometry changed from 0.7 to 1.8 while the  $H^+$ /acetate value increase from 0.9 to 1.9 at pH 7.5. The authors stated that the increase in  $H^+$ /product stoichiometric ratio under alkaline conditions demonstrated that *L. plantarum* co-excreted more protons with end products to maintain intracellular pH homeostasis and generate proton gradients.

The maintenance of pH homeostasis of *L. plantarum* was examined during growth in the presence of glucose and pyruvate (86). The energy derived from the metabolism of glucose generated a constant  $\Delta p$  of about -120 mV regardless of medium pH. The  $\Delta pH$  increased with the decreasing external pH while the  $\Delta \Psi$  decreased correspondingly, so that the  $\Delta p$  remained constant. In medium at low pH, energized cells rapidly transported and accumulated pyruvate, but did not do so when the cells were deenergized by nigericin. This confirmed that pyruvate

was actively transported. The internal pH of cells that accumulated pyruvate immediately decreased from pH 6.3 to 5.5 due to the cotransported protons. The conversion of pyruvate to acetoin, instead of to acidic end-products, caused the internal pH to increase gradually.

Fermentation patterns of *Sarcina ventriculi* were investigated during glucose metabolism in unbuffered batch cultures (31). The culture shifted from hydrogen-acetate production to ethanol production as the medium pH declined from 7.0 to 3.3. At a constant pH of 3.0, acetate production ceased when the concentration of acetate in the medium reached 40 mM, while at pH 7 acetate production continued throughout the entire growth time course. As the pH of the medium declined from 7.0 to 3.0, the  $\Delta\text{pH}$  increased from 0.1 to 1.3 and the  $\Delta\Psi$  decreased from -67 mV to -38 mV; the magnitude of  $\Delta p$  increased as the medium pH decreased. The addition of acetic acid, but not hydrogen or ethanol, inhibited growth and resulted in  $\Delta p$  dissipation and internal acetic acid accumulation. The authors suggested that the ability of *S. ventriculi* to shift to ethanol production and continue to ferment glucose while cytoplasmic pH values were low adapted this organism for growth at low pH.

The proton motive force was involved in the regulation of the transition from acetogenic to solventogenic fermentation in *Clostridium acetobutylicum* as well (42). The extracellular pH of the culture was varied from pH 6.5 to 4.5 and the  $\Delta\text{pH}$ ,  $\Delta\Psi$  and fermentation products were measured. The internal pH dropped from 6.7 to 6.0 when the external pH changed from 6.5 to 5.5, but there was no further decline in internal pH even when the medium pH decreased to 4.5. The  $\Delta\Psi$  decreased as the external pH decreased; at pH 6.5,  $\Delta\Psi$  was -90 mV and at pH

4.5 no  $\Delta\Psi$  was detected. The corresponding  $\Delta p$  values were -106 mV at pH 6.5 and -102 mV at pH 4.5. The ability of *C. acetobutylicum* to maintain a high internal pH even at pH 4.5 points to a very efficient deacidification system. This system was presumed to be responsible for the shift from acetogenesis to solventogenesis when the medium pH dropped below 5.5.

The shift to solvent formation appeared to occur between pH 5.0 and 5.5 (42). Acetone and butanol were the predominant products at low pH but were present in only trace amounts at high pH, while butyrate and acetate were the major products formed at high pH. The researchers noted that hydrogenase, which is responsible for the disposal of excess hydrogen as hydrogen gas, was pH-sensitive. The loss of this activity may result in more reducing power becoming available for solvent formation. In addition, the enzyme acyl coenzyme A transferase, which converts fatty acids to the corresponding coenzyme A derivative, showed increased activity at low pH. Solvent formation appeared to allow *C. acetobutylicum* to maintain internal pH homeostasis.

### **Acidophiles**

An alternative approach to studying the mechanism of acid inhibition is to examine organisms that tolerate high levels of acid. Acidophiles, i.e. organisms whose optimal pH is generally between 2 and 4, have been the subject of several studies (1, 14, 33). Respiring acidophiles maintain a large  $\Delta p$ , which consists of a large  $\Delta pH$  and a small  $\Delta\Psi$ . The  $\Delta\Psi$  is reversed from that of other organisms (nonacidophiles), i.e. positive inside the cell. The  $\Delta\Psi$  most likely plays a role in the maintenance and perhaps even generation of  $\Delta pH$ . These organisms can



tolerate acidic media because they respire rapidly, thereby ejecting protons. In addition, the cells do not produce organic membrane-permeable fermentation end-products (48).

In a study of sensitivity to anions and organic acids of the acidophile *Thiobacillus ferrooxidans*, Alexander *et al.* (1) suggested that the toxicity of weak acids could be attributed to their accumulation in the cell matrix in response to the pH difference between the cytoplasm and the supporting medium. This accumulation led to cytoplasmic acidification that resulted in inhibition of the respiratory chain and cessation of growth.

Goulbourne *et. al* (33) measured the proton motive force of active and starved cells of PW2, a heterotrophic, mesophilic acidophile. The cells were starved in a mineral salts medium lacking citrate and magnesium. The starved cells possessed no respiration, no cellular ATP and no ATPase activity, yet they maintained a large  $\Delta\text{pH}$ . This organism was able to maintain a passive  $\Delta\text{pH}$  by the buildup of the counterforce,  $\Delta\Psi$ , in the same magnitude, but opposite polarity (interior positive). The role of this electrical potential force was to prevent a net  $\text{H}^+$  influx and thus help to maintain the cytoplasmic pH.

In activated cells metabolic energy was available, and respiration-dependent  $\text{H}^+$  extrusion offset the  $\Delta\Psi$  (33). Eventually a steady state was achieved in which  $\text{H}^+$  influx was equal to  $\text{H}^+$  efflux. The addition of 1.3 mM azide to these cells caused a net  $\text{H}^+$  influx of 14.4 nmol per mg of protein, which should be sufficient to lower the internal pH to 2. However, this concentration had no effect on the cytoplasmic pH. The cells must have resisted acidification by their cytoplasmic buffering capacity. The authors concluded that the mechanism that

linked  $H^+$  influx with the rise in  $\Delta\Psi$  as well as the cytoplasmic buffering capacity played a central role in the acidophile's mode of existence.

Some believe that  $\Delta pH$  is maintained in acidophiles by Gibbs-Donnan equilibrium (14, 48). According to this mechanism,  $H^+$  entry is prevented by a high intracellular concentration of cations to which the membrane is impermeable. Thus, even when treated with an uncoupler, the cell is able to maintain the  $\Delta pH$  by a mechanism that does not require an immediate source of metabolic energy.

### **Acid tolerance response**

It has been found that some organisms are able to increase their resistance to extreme acid conditions through previous exposure to mildly acidic conditions. Examination of this response may offer insight into acid tolerance. Foster and Hall (27) discovered that when logarithmically growing *Salmonella typhimurium* cells (pH 7.6) were shifted to pH 5.8 by adding hydrochloric acid to the medium for one culture doubling time (preshock), the cells were 100 to 1,000 times more resistant to subsequent strong acid challenge (pH 3.3) than were unadapted cells shifted directly from 7.6 to 3.3 (acid shock). This response was called the acidification tolerance response (ATR).

By measuring the internal pH of adapted and unadapted cultures at pH 3.3, it was found that the ATR system produced an inducible pH-homeostatic function (28). The adapted culture was able to maintain an internal pH of 5.2 to 5.4 while the internal pH of the unadapted culture was 4.2 to 4.6. In addition, the  $H^+$ -ATPase was found to play an important role in ATR (27, 28). Mutants lacking this activity were unable to display an ATR and were extremely acid-sensitive.

The ATR required the synthesis of preshock proteins and appeared to be a specific defense mechanism for acid, since no cross-protection was noted for hydrogen peroxide, SOS (the multifunctional DNA damage repair system), or heat shock (27). However, other researchers found that adapted cultures of *S. typhimurium* had an increased tolerance towards heat, salt, an activated lactoperoxidase system, and the surface-active agent crystal violet (53). These researchers found that acid adaptation increased the cell surface hydrophobicity.

The analysis of two-dimensional SDS-PAGE autoradiographs revealed six repressed and 12 induced membrane proteins in adapted cultures. Leyer and Johnson (53) found that the lipopolysaccharide component of the membranes appeared to be unaltered and thus, the induced outer membrane proteins were thought to be responsible for the change in surface properties.

During acid shock, *S. typhimurium* induced the synthesis of 37 proteins and repressed 15 (29). All but four were distinct from the preshock ATR proteins. Acid shock alone did not protect the culture against acidic conditions. Inhibiting protein synthesis with chloramphenicol prior to preshock or acid shock revealed that acid shock proteins did not appear to contribute to acid survival in medium even at pH 4.3, however, preshock proteins were necessary for resistance. These observations indicated that the induction of acid shock and preshock ATR proteins were separate processes requiring separate signals. The organism required both systems to survive extreme acid conditions. Foster (29) proposed a two-stage process that allowed the organism to phase in acid tolerance as the environmental pH became progressively more acidic.

A recent study showed that stationary-phase cells of *S. typhimurium* had two additional acid tolerance responses (52). One was a pH-dependent system that was distinct from log-phase ATR. Stationary-phase cells with a final pH of 4.3 survived a subsequent 4-h pH 3.0 challenge at least 1000 times better than stationary-phase cells that had a final pH of 7.3. The stationary-phase ATR induced fewer proteins than did log-phase ATR. The other system of acid resistance was not induced at low pH, but appeared to be part of a general stress resistance induced by stationary phase. It was determined that although all three systems were distinct from each other, together they afforded maximum acid resistance for *S. typhimurium*.

### **Production of acid-tolerant organisms**

Many researchers are interested in increasing end-product tolerance of microorganisms. These tolerant strains could be important to several industrial fermentations for production of fuel additives (butanol and ethanol) and organic acids (lactic and propionic acids). Lin and Blaschek (54) used serial enrichment to obtain a derivative of *Clostridium acetobutylicum* that grew at concentrations of butanol that prevented growth of the parent strain. At 15 g/L of butanol, the wild-type strain demonstrated a negative growth rate while the mutant (SA-1) grew at a rate that was 66% of that of the uninhibited control. When the parent and SA-1 strain were grown in batch fermentation with 6% extruded corn broth, SA-1 had a significantly faster generation time than the parent strain (32.8 min versus 57.9 min), a 9- to 12-h earlier onset of butanol production, better carbohydrate utilization (88.1% versus 82.3%) and a higher final butanol concentration (8.6 g/L

versus 6.8 g/L). In addition, SA-1 demonstrated higher conversion efficiency to butanol than did the wild-type strain.

Jimenez and Benitez (44) formed hybrids between naturally occurring and laboratory wine yeast strains to increase genetic variability and improve ethanol tolerance of the strains. The hybrids were cultivated in pH-controlled continuous culture with increasing ethanol concentration. The fastest growing, most ethanol-tolerant strains were selected by this system.

In another experiment, *Streptococcus mutans* was shown to increase its acid tolerance when grown in continuous culture at low pH (5). Cells harvested from the acidified cultures had a lower pH minimum for glycolysis as well as increased ATPase activity. However, this adaptation was found to be reversible over a number of generations, and thus did not seem to be due to selection of variants in the population with greater constitutive acid resistance. The authors suggested that increased ATPase activity was the major factor in the cultures' increased acid tolerance.

Woskow and Glatz (91) developed an acid-tolerant strain of *Propionibacterium acidipropionici* by a serial enrichment process. This strain (P200910) showed significantly higher specific growth rates than its parent strain (P9) at 1 to 7% propionic acid. However, in laboratory media without propionic acid, P200910 had a longer lag time and grew to a lower final cell density than did P9. In batch fermentation, P200910 produced more propionic and acetic acid per gram biomass than P9 (0.91 versus 0.50 for propionic acid and 0.21 versus 0.13 for acetic acid). The variant exhibited a propionic acid production pattern

typical of non-growth-associated product formation, while the parent followed a typical growth-associated pattern.

When both strains were used in fed-batch semi-continuous fermentations, much higher final concentrations of biomass and organic acids were obtained for both strains than in simple batch fermentations (91). After 5 days of incubation, P9 had produced 26 g/L of propionic acid and 0.41 g of propionic acid per g of biomass, while P200910 produced 38 g/L and 0.81 g of propionic acid per g of biomass. Rates of propionic acid production decreased for both strains after this time. However, P200910 continued to use glucose at a high rate throughout the fermentation. These observations led the authors to suggest that the acid-tolerant strain may expend energy to rid itself of excess acid.

The variant did not differ from its parental strain in morphology, gram reaction, fermentation of sucrose, maltose and mannitol, reduction of nitrate or pigment production (91). However, changes in lipid composition of the variant from more branched-chain fatty acids to straight-chain fatty acids did occur. The authors suggested such changes may have resulted in a less fluid, less permeable membrane.

The precise method by which short-chain organic acids inhibit microbial growth is still unresolved. Studies discussed here were based on different hypotheses and it appears that no single mechanism applies to all organisms. Further examination and characterization of acid-tolerant variants and their parental strains will lead to a greater understanding of the mechanism of acid inhibition.

## RESPONSE OF CULTURES OF *PROPIONIBACTERIUM* TO ACID AND LOW PH: TOLERANCE AND INHIBITION

A paper prepared for submission to the Journal of Industrial Microbiology

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### Abstract

*Propionibacterium* strains were screened for acid production and final pH achieved on glucose, fructose, and maltose as primary carbon source. On average, the *P. acidipropionici* strains produced more acid and achieved lower final pH values than did the other species. Six strains, which consisted of three *P. acidipropionici*, one *P. jensenii*, and two *P. thoenii* strains, were identified as potential acid- and low-pH-tolerant strains. The lowest pH values at which the strains could initiate growth and remain viable were determined for the six strains with lactic, hydrochloric, and propionic acid as the acidulants. For all three acids, the *P. jensenii* and *P. thoenii* strains initiated growth and survived at lower pH values than did the *P. acidipropionici* strains. Thus, the ability to produce large amounts of acid or achieve low final pH values was not directly related to a strain's ability to initiate growth or survive in low-pH conditions. In addition, the strains were not inhibited by increased lactate concentrations (up to 180 mM) at neutral pH.

## Introduction

The propionibacteria are gram-positive, nonmotile, pleomorphic rods that are anaerobic to aerotolerant [8] and produce propionic acid, acetic acid, and carbon dioxide from the metabolism of sugar and/or lactate [25]. They have been isolated from soil, silage, rumen, raw milk, Swiss cheese, and other dairy products [21, 25].

Propionibacteria have traditionally been used in the dairy industry as starter cultures. In addition, the propionibacteria can be used to produce valuable products such as propionic acid, which is an important preservative for foods and grains [13, 21]. Propionate inhibits growth of molds, bacteria, and yeasts; it is biostatic in dilute aqueous systems and biocidal at higher concentrations [13].

Currently commercial production of pure propionic acid is chiefly by petrochemical routes. Fermentation has not been used commercially primarily because propionibacteria grow slowly and produce relatively low concentrations of organic acids. Also, the costs of separation and concentration of the acids make recovery difficult and expensive [21].

A major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid [11, 25]. Accumulation of end products causes a continuous decrease in both specific growth rate and the further production of the product [11]. To overcome this end-product inhibition,



either improvements in the fermentation process to avoid product accumulation can be made, or an acid-tolerant variant of the producer strain can be developed.

A strain of *Propionibacterium* that could tolerate high concentrations of organic acids and low pH could be of economic importance as a more productive strain for industrial fermentations or as part of a silage inoculum or probiotic feed additive. One objective of this work was to identify strains that produced high amounts of acid and can grow at low pH (pH 5.0 or lower) on various carbon sources. A second objective was to determine the lowest pH (adjusted with lactic, propionic, or hydrochloric acid) at which growth occurred as well as the pH at which significant loss of viability occurred.

## **Materials and Methods**

### **Strains and culture maintenance**

*Propionibacterium* strains that had not been previously screened for acid production as well as strains previously identified as good acid producers [1] were obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Strains were classified according to Bergey's Manual of Systematic Bacteriology [8]. The strains were grown in sodium lactate broth (NLB) which contained 1% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA) and 0.6% Tween 80 (Fisher). Stock cultures were maintained at -80°C in NLB supplemented with

20% glycerol. Working cultures were maintained on sodium lactate agar plates (NLA; NLB + 1.5% agar, Difco), grown for 5 days at 32°C in an anaerobic environment in GasPak jars (BBL) with the GasPak system, stored at 4°C, and transferred at approximately one-month intervals.

Isolated colonies from working plates were inoculated into screw-capped test tubes containing 10 ml NLB and incubated at 32°C for 48 h. At least 3 sequential transfers were made by transferring 0.1 ml of culture to a test tube containing 10 ml NLB and incubating at 32°C for 24 h. The cells were harvested in the exponential phase (absorbance of approximately 0.6 at 600 nm) for use in experiments.

### **Culture screen**

Cultures were spotted as 5- $\mu$ l drops (approximately  $10^6$  cells) onto bromocresol agar (BCP agar) to test for acid production as measured by pH change [22]. The plates were incubated at 32°C for 5 to 7 days in the GasPak system. Acid production was semiquantitatively determined by measuring the diameter of the zone of yellow around the colony. To test for tolerance to lactate at both neutral and acidic conditions, cultures were also inoculated onto NLA supplemented with different concentrations of sodium lactate (2 to 3.5%) at pH 7.0 or adjusted to pH 5.5 or 5.0 with lactic acid (Fisher) as the acidulant. Plates were incubated at 32°C for 5 to 28 days in the GasPak system.

The best acid-producing strains identified in the above screening experiments were grown (1% inoculum) in duplicate in 15-ml screw-capped test tubes with 10 ml modified NLB in which the sodium lactate was replaced by 1%

glucose, maltose, or fructose. The tubes were incubated for 96 h at 32°C, the final pH measured (Corning Science Products, Corning, NY), and propionic and acetic acid concentrations determined by HPLC.

### **Minimum and lethal pH determinations**

Cultures were grown in 15-ml screw-capped test tubes containing 10 ml NLB adjusted to pH values ranging from 6.5 to 3.75 with lactic, propionic, or hydrochloric acid (Fisher). The experiment was conducted by using a Randomized Block Design containing 2 blocks, each of which consisted of a separate batch of medium, with 2 replicates in each block [24]. Test tubes were inoculated with approximately  $10^6$  or  $10^4$  cells/ml. The cultures were incubated at 32°C for 7 days and examined each day for the presence of growth, as indicated by visible turbidity. If no growth appeared after 48 h, a 0.1-ml sample of the culture was used to inoculate a fresh tube of NLB at pH 7.0. These cultures were incubated at 32°C for 7 days and examined each day for growth.

### **Growth curves**

Strains P9, P127, and P200910 were grown in 250-ml screw-capped Erlenmeyer flasks containing 100 ml of NLB and various concentrations of lactic acid adjusted to both neutral and acidic pH values. The cultures were incubated at 32°C and sampled at timed intervals. At each sampling time, absorbance, pH, and concentrations of lactate, propionic, and acetic acid were determined. Specific growth rates ( $\mu$ ) during exponential growth were determined from the slope of the straight line portion of the semilogarithmic plot of absorbance versus time [15].

### **Analysis of products**

Lactate, propionate, and acetate were separated by a high-performance liquid chromatography (HPLC) system that consisted of a model 501 pump (Waters, Division of Millipore, Milford, MA) and an HPX-87H column (BioRad, Richmond, CA) operated at 65°C with 0.012N H<sub>2</sub>SO<sub>4</sub> (pH 2.0) as the mobile phase at a flow rate of 0.8 ml min<sup>-1</sup> and an injection volume of 20 µl. Peaks were detected with a Waters differential refractometer (model R401). Samples for analysis were centrifuged to remove the cells, filtered through 0.2-µm filters, and stored at -20°C before analysis. A Maxima 820 software program (Waters) was used to analyze the data and plot the chromatograms. The product concentration was calculated by comparing the peak areas with those of external standards (Aldrich Chemical Co., Milwaukee, WI).

### **Statistical analysis**

An analysis of variance was performed by using the General Linear Model of SAS [23]. Differences with  $P < 0.0001$  were considered significant only if they were at least 0.1 pH unit. This pH difference was considered significant because the strains responded to pH values that differed by 0.1 pH unit, i.e. strains could grow at pH 5.0 but not at pH 4.9.

## **Results and Discussion**

### **Strain selection**

The initial objective of this work was to select the most acid- and pH-tolerant strains in our culture collection. Strains were selected based on their ability to metabolize all three carbon sources, their tolerance to 3.5% lactate, and the amount of acid produced. Acid production was estimated by the size of the zone of yellow around the colony on BCP agar. All strains were able to grow in the presence of 3.5% lactate and on pH 5.5 agar plates (data not shown). However, no strains were able to grow on pH 5.0 agar, even after a 4-week incubation in GasPak jars. Seventeen strains, including seven strains previously identified as good acid producers on maltose [2], were chosen for further study primarily based on the acid zone size on BCP agar.

### **Propionic and acetic acid production**

The ability of propionibacteria to produce propionic and acetic acids with maltose, fructose, or glucose as the carbon source was tested by measuring the acids produced and pH achieved after 96 h in broth culture (Table 1). In general, the final pH was lowest when glucose was the carbon source (pH 4.28  $\pm$  0.18). With fructose as carbon source, the average final pH was higher (4.39  $\pm$  0.24); on maltose it was higher still (pH 4.53  $\pm$  0.38). The final pH differed the most among strains grown in maltose broth, as indicated by the largest standard deviation (0.19 pH unit versus 0.09 pH unit for glucose and 0.12 pH unit for

fructose). This indicates that strains of propionibacteria differ significantly in their ability to transport and/or hydrolyze maltose.

The average propionic:acetic acid ratios were approximately 4:1 for cultures grown on glucose and fructose and 3:1 for those grown on maltose (Table 1). Significant differences among the strains in the amounts and ratios of acids produced are consistent with previous observations that *P. acidipropionici* produced significantly different concentrations of propionic and acetic acids when grown on lactate, glucose, and lactose [18]. Variations in the ratios of acids produced by cultures of propionibacteria have been credited to the reactions of the citric acid cycle [6, 25], to aspartate metabolism [5, 6], and to polysaccharide production [7]. The utilization of aspartate varies among species and strains and shifts the balance from propionate production to acetate and carbon dioxide formation to maintain the redox balance of the cell [5, 6]. The formation of polysaccharides during glucose and lactose fermentation by propionibacteria alters the acid ratio by increasing the formation of acetate and carbon dioxide without any associated propionate production [7]. Thus, some of these strains may have produced different acid ratios because of these different reactions.

Strains of *P. acidipropionici* seemed to be the most acid-tolerant; 14 of the 17 selected strains were members of this species. These strains also produced higher amounts of propionic and acetic acids on all three carbon sources than did strains of *P. thoenii* and *P. jensenii*. Previous work in our laboratory found *P. acidipropionici* strains were the best acid producers on sodium lactate and lactose as well [2].

Among the strains studied, the ability to produce the most acid on a particular carbon source did not necessarily correlate with attaining the lowest final pH at the end of growth. Strain P9 and P86 reached the lowest pH with glucose as the carbon source (pH 4.17 and 4.18, respectively), but strain P200910, a propionate-tolerant variant of P9 developed in our laboratory [27], produced the most propionic and acetic acids (8.31 and 2.75 g l<sup>-1</sup>, respectively). With fructose as the carbon source, P9 reached the lowest final pH (4.29), but P86 produced the most propionic acid (5.68 g l<sup>-1</sup>), and P131 produced the most acetic acid (1.52 g l<sup>-1</sup>). In maltose broth, strain P200910 reached the lowest pH (4.25) and produced the most propionic acid (5.31 g l<sup>-1</sup>), but P127 produced the most acetic acid (1.78 g l<sup>-1</sup>). The final pH achieved may depend on the culture's ability to conserve nitrogen-containing compounds in the medium that contribute to the buffering capacity [2]. Propionibacteria have been shown to metabolize several amino acids, especially aspartate, alanine, serine, and glycine [5, 6, 16]. However, these investigators observed that the specific activities of the enzymes involved in amino acid degradation (e.g. aspartase and alanine dehydrogenase) differed significantly among strains studied. In addition, some strains did not degrade amino acids.

Six strains (P9, P20, P126, P127, P155, and P200910) were selected for further study based on their low average final pH on all carbon sources, the amounts of acids produced, and other significant characteristics (i.e. P126 and P127 produce bacteriocins [10, 19] and P200910 is a propionate-tolerant variant of P9). The final pH was considered most important because low pH seemed to be the most inhibitory factor, i.e. no strains could grow on solid medium acidified

to pH 5.0. In addition, the original goal was to select a strain that would be useful as a silage inoculant and thus must be tolerant to high lactate and low pH. Each strain chosen reached an average pH <4.4.

In an effort to isolate pH-tolerant variants, strains were grown in NLB acidified to pH 5.0 with lactic acid. The cultures were incubated until visible turbidity appeared and then were transferred (1% inoculum) to another test tube containing pH 5.0 NLB. However, after 3 to 4 such transfers the strains were no longer able to grow. The strains were also streaked across pH gradient plates and incubated at 32°C in GasPak jars for 7 to 28 days. It was hoped that the most tolerant cells in the population would be selected and pushed to greater tolerance; however, the strains would not grow at pH values lower than pH 4.75 even after 28 days.

In another attempt to select the strain that could best tolerate low pH, the strains were inoculated as a mixed culture in a fermenter at pH 5.0. The intention was to start fermentation as a batch culture at pH 5.0 and then begin slow medium addition with gradual decrease in pH. However, the strains did not grow in batch culture after 48 hours, (i.e. no change in absorbance). The same results were obtained three times. Viable counts were essentially unchanged after 48 hours. Thus, it appears that the cells were able to survive even though they did not divide and grow. Previous researchers had observed that the pH optima of enzymes are narrow. This allows internal enzymes to be active only at a pH above a certain critical value with this threshold pH value depending on the organism [3, 13]. Our observation that there is a difference between the pH value at which cells could multiply and the value at which cells were killed may suggest that the biosynthetic



enzymes of the cells were more pH-sensitive than enzyme(s) essential for cell survival.

### **Minimum growth and lethal pH**

To investigate further this disparity between lethal pH and pH for growth, the minimum growth pH was determined as the lowest pH at which the culture developed visible turbidity. The lethal pH, the highest pH at which significant loss of culture viability occurred, was determined as the pH value at which the cells could not be recovered at optimum conditions (i.e. pH 7.0). The minimum growth pH and lethal pH values were determined for each strain in broth acidified with lactic, propionic, or hydrochloric acid, inoculated with either  $10^4$  or  $10^6$  cells/ml (Figures 1 and 2).

There were significant differences among the abilities of the strains to grow and survive in each acid and at each inoculum level; no single strain had the lowest minimum growth pH and lethal pH values for all three acids at both inoculum levels (Figures 1 and 2). However, strain P127 could grow at the lowest pH values with all three acids at the higher inoculum level: pH 4.95 with lactic acid, pH 4.73 with hydrochloric acid, and pH 4.94 with propionic acid (Figure 1). Also, strain P200910 seemed to be the most sensitive to pH when inoculated at  $10^4$  cells/ml; it had the highest lethal and minimum growth pH values with all three acids (Figure 2).

When averaged over all strains, inoculum level had the most significant effect on minimum growth pH values of the cultures. On average when cultures were inoculated at  $10^6$  cells ml<sup>-1</sup>, they grew at pH values that were 0.34 pH unit

lower than when inoculated at  $10^4$  cells  $\text{ml}^{-1}$  (Figure 1). In addition, cultures were able to survive at pH values that were 0.13 pH units lower when inoculated at  $10^6$  cells/ml versus  $10^4$  cells  $\text{ml}^{-1}$  (Figure 2). This difference was greatest for NLB acidified with propionic acid. However, the effect of inoculum level differed between the strains (Figure 3). Inoculum size had a greater effect for *P. acidipropionici* strains than for *P. jensenii* or *P. thoenii* strains. Previous researchers [2] found that inoculum size significantly influenced biomass and acid production as well. In addition, larger inocula have been found to increase the “adaptability” of the propionibacteria and result in a shorter lag phase and better growth [26].

The nature of the acid also had a significant effect on the minimum growth and lethal pH values. Cultures grown in broth with hydrochloric acid as the acidulant were able to grow at pH values that were 0.21 pH unit lower than for cultures grown in the presence of organic acids. Cultures could survive in broth acidified with hydrochloric acid to a value 0.34 pH unit below the lethal pH values of broth containing lactic acid or propionic acid as the acidulant. This is consistent with previous observations that organic acids are more inhibitory than are inorganic acids [9]. The antimicrobial action of organic acids has been attributed both to a generalized inhibition due to low pH and a specific inhibition due to the action of the anion, while inorganic acid inhibition is solely due to low pH [9].

Propionic acid was the most inhibitory of the three acids. The average minimum growth pH was 5.29 and the average lethal pH was 4.31 across all strains for this acid. In comparison, the average minimum growth pH values were 5.13 and 5.00 and the lethal pH values were 4.13 and 3.87 for lactic and

hydrochloric acids, respectively. Propionic acid has been found to be more effective than lactic acid in inhibiting salmonellae [4] and acid-tolerant yeasts that cause food spoilage [20].

Although the *P. acidipropionici* strains (P9, P155, and P200910) produced lower final pH values and on average more acid than the *P. thoenii* (P20 and P127) and *P. jensenii* (P126) strains in glucose, fructose, and maltose broth (Table 2), they did not initiate growth or survive at lower pH values (Figures 1 and 2). The average minimum growth pH values of the *P. acidipropionici* strains were 0.17 pH unit higher than those of the other strains. The ability to achieve a low final pH or produce large amounts of propionic acid may not be directly related to the ability to initiate growth at low pH or in the presence of propionic or other acids.

The difference between tolerance of the acid produced by a culture and the ability to initiate growth under similar conditions of acid concentration and low pH is illustrated further in Tables 2 and 3. When inoculated at  $10^6$  cells/ml into medium acidified with propionic acid, strains could not initiate growth below approximately pH 5.0, which corresponded to 25 mM propionic acid (Table 3). However, all of these strains reached final pH values <4.4, and produced propionic acid in concentrations ranging from 41 to 112 mM (Table 2). This may suggest that as a culture grows it adapts to its environment and thus can tolerate conditions in which it could not initiate growth. Late-log and stationary phase cells of *E. coli* O157:H7 were more acid-tolerant than mid-log cells [1]. In addition, stationary phase cells are more resistant to a variety of chemical and physical

challenges than are cells in other phases of growth. This is due to lower enzyme activity and thicker cell walls during stationary phase [16].

When the medium is acidified with an organic acid, inhibition may be due primarily to the concentration of the anion or to the hydrogen ion. To differentiate between these, all strains were grown in pH 7.0 NLB with increased lactate concentrations from 90 to 180 mM and propionate concentrations from 50 to 250 mM. All strains were able to grow in the presence of 180 mM lactate (data not shown); this was equivalent to the concentration of lactate used to adjust the medium to pH 4.0, at which growth was prevented. Thus, the strains were inhibited by the hydrogen ion concentration of the medium, not the lactate ion.

However, strains P20, P126, and P127 were more sensitive to the propionate anion than were the *P. acidipropionici* strains. Strains P20, P126, and P127 were inhibited by propionate concentrations greater than 150 mM, while strains P9, P155, and P200910 were able to grow in 250 mM propionate (data not shown). These results suggest that there is a significant difference between tolerance to lactate and propionate; this agrees with previous observations that propionic acid was more inhibitory to these strains than was lactic acid (Figures 1 and 2). The inhibition appears to be due to the specific action of the propionate anion, at least for strains P20, P126, and P127. Furthermore, the results confirm our conclusion that the ability to tolerate or produce high concentrations of propionate is not directly related to the ability to tolerate or initiate growth at low pH.

### Effect of lactic acid on propionic and acetic acid yields

To determine if the lactic acid concentrations at acidic or neutral pH values affected the propionic and acetic acid yields (g acid produced per g substrate consumed), strains P9, P127, and P200910 were inoculated into broth containing increased lactic acid concentrations. The pH values were 7.0, 5.0 (the minimum growth pH for these strains), 4.75, and 4.25 (the lethal pH for these strains). The corresponding lactate concentrations were 55, 70, 90, and 120 mM, respectively.

As expected, none of the strains were able to grow at pH 4.75 or 4.25. Although the strains could survive at pH 4.75, they did not produce any measurable propionic or acetic acid. Thus, the ability to survive was not contingent on acid production. This suggests that these strains are able to remain viable even when they are not actively metabolizing.

Propionic acid yield decreased with increasing lactate concentrations for all strains (Table 4). At all lactate concentrations, strain P127 had the highest propionic acid yields while P200910 had the lowest yields. Low pH did not have a significant effect on propionic or acetic acid yields for P9 or P127. Previous research indicated that propionic acid yield of *P. acidipropionici* increased with decreasing pH but that acetic acid yields were not affected by pH during growth on lactose and glucose [12, 18]. The authors found that at low pH (pH 4.5-5.0) the culture produced less succinate and pyruvate; with less carbon diverted to other products, more propionic acid could be produced. In the current study we found no significant levels of succinate at any of the lactate concentrations studied, regardless of the pH value. This is consistent with previous observations of *P. acidipropionici* fermentation of lactate [18].

As previously observed for propionibacteria [18], the specific growth rates ( $\mu$ ) were significantly slower at pH 5.0 than at neutral pH (Table 4). Low pH had the most significant effect on P200910, which did not grow at pH 4.93. Strain P200910 appeared to have a distinct pH threshold; it was able to grow at pH 5.08 (Figure 1) but did not grow in medium that was 0.15 pH unit lower (Table 4). These results agree with the observation that biosynthetic enzymes have narrow pH optima [3, 13].

Increased lactate concentrations at neutral pH did not have a significant effect on the specific growth rates of the cultures; all cultures grew at similar growth rates. This indicates that inhibition at low pH was not due to the lactate anion, but to proton concentration.

The initial goal of this study was to identify potential acid- and low-pH-tolerant variants which could be used as silage inocula or probiotic feed additives. We proposed that strains that produced large amounts of acids and obtained a low final pH would be the best strains to select. However, there appeared to be a distinct difference between ability to tolerate low pH (hydrogen ions) versus high concentrations of the acid anion. Thus, isolation and/or development of an acid- and low-pH-tolerant variant seems unlikely; tolerance to low pH and acid anions appeared to be two different mechanisms. Indeed, the propionate-tolerant variant of P9, P200910, had been isolated after repeated exposure to increasing propionate concentrations at neutral pH [27], but it showed the least tolerance to low pH. Strains P20, P126, and P127 are potential strains for low-pH-tolerant variants while strains P9 and P155 have potential as acid-tolerant variants which could be used as more productive strains for industrial fermentations.

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Table 1. Acid production and final pH achieved by cultures of propionibacteria grown on various substrates for 96 h. Values are the average of duplicate cultures with standard error values <0.12.

Species	Strain	Glucose			Fructose			Maltose		
		pH	P.A. <sup>a</sup>	A.A. <sup>b</sup>	pH	P.A. <sup>a</sup>	A.A. <sup>b</sup>	pH	P.A. <sup>a</sup>	A.A. <sup>b</sup>
<i>P. acidipropionici</i>	P2	4.30	5.10	1.01	4.31	5.58	1.50	4.67	2.76	1.12
	P3	4.37	4.49	0.81	4.37	5.31	1.34	4.88	2.42	0.90
	P5	4.38	2.11	0.60	4.81	2.12	0.60	4.72	2.30	1.03
	P9	4.17	4.31	0.91	4.29	5.16	1.27	4.26	4.15	1.29
	P50	4.47	3.31	1.22	4.57	3.40	1.31	4.55	2.84	0.98
	P78	4.44	4.09	1.32	4.33	4.89	1.12	4.63	2.77	1.02
	P86	4.18	6.39	1.28	4.31	5.68	1.38	4.74	3.71	1.25
	P125	4.28	5.29	1.10	4.40	5.20	1.26	4.46	3.41	1.16
	P131	4.21	4.92	1.27	4.35	4.65	1.52	4.54	3.24	1.12
	P133	4.28	6.13	1.26	4.38	5.52	1.42	4.82	2.36	0.92
	P134	4.29	4.70	1.12	4.41	4.78	1.32	4.53	3.51	1.22
	P155	4.22	4.75	0.92	4.33	5.57	1.05	4.34	4.25	1.24
	P156	4.22	4.69	0.95	4.32	5.28	1.14	4.44	4.69	0.95
	P200910	4.21	8.31	2.75	4.35	5.27	1.14	4.25	5.31	1.50
<i>P. thoenii</i>	P20	4.21	5.47	1.37	4.40	5.02	1.36	4.46	3.06	1.17
	P127	4.26	3.82	0.95	4.35	4.46	1.30	4.37	4.57	1.78
<i>P. jensenii</i>	P126	4.26	3.79	0.93	4.41	4.01	1.09	4.39	3.93	1.56
Average		4.28	4.80	1.16	4.39	4.82	1.24	4.53	3.49	1.19
St. Dev.		0.09	1.36	0.46	0.12	0.92	0.23	0.19	0.89	0.24

<sup>a</sup> P.A. = propionic acid in g l<sup>-1</sup>

<sup>b</sup> A.A. = acetic acid in g l<sup>-1</sup>

Table 2: Final pH and concentration of propionic acid (mM) produced in broth culture with various carbon sources when inoculated at  $10^6$  cells  $\text{ml}^{-1}$ .

Strain	Glucose		Fructose		Maltose	
	pH	P.A. <sup>a</sup>	pH	P.A. <sup>a</sup>	pH	P.A. <sup>a</sup>
P9	4.17	58	4.29	70	4.26	56
P20	4.21	74	4.40	68	4.46	41
P126	4.26	51	4.41	54	4.39	53
P127	4.26	52	4.35	60	4.37	62
P155	4.22	64	4.33	75	4.34	57
P200910	4.21	112	4.35	71	4.25	72

<sup>a</sup> P.A. = propionic acid in mM

Table 3: Minimum growth and lethal pH values and corresponding concentrations of propionic and lactic acids (mM) for cultures inoculated at  $10^6$  cells  $\text{ml}^{-1}$ .

Strain	Propionic acid				Lactic acid			
	Minimum pH	Minimum mM	Lethal pH	Lethal mM	Minimum pH	Minimum mM	Lethal pH	Lethal mM
P9	5.07	25	4.27	160	5.08	70	4.04	137
P20	5.07	25	4.15	229	4.95	73	3.92	145
P126	5.07	25	4.21	192	5.08	70	3.92	145
P127	4.94	31	4.26	165	4.95	73	4.10	135
P155	5.07	25	4.32	138	4.95	73	4.10	135
P200910	5.07	25	4.15	229	5.08	70	4.10	135

Table 4. Yield (g acid produced/g lactate consumed) of propionic and acetic acids of cultures grown in media with various lactate concentration at different pH values. Values are the average of duplicate cultures with standard error values <0.15.

Strain	Lactate (mM)	Initial pH	$\mu^a$ (h <sup>-1</sup> )	$Y_p^b$ (g/g)	$Y_a^c$ (g/g)	Lowest pH	Final pH
P9	55	6.88	0.15	0.55	0.31	5.58	5.78
	70	6.81	0.15	0.56	0.21	5.59	5.87
	70	4.94	0.044	0.51	0.26	4.90	5.27
	90	6.74	0.15	0.55	0.30	5.55	5.76
	120	6.45	0.15	0.52	0.27	5.48	5.77
P127	55	6.88	0.13	0.50	0.31	5.53	5.84
	70	6.82	0.12	0.51	0.31	5.54	5.88
	70	4.95	0.053	0.54	0.29	4.78	5.27
	90	6.74	0.12	0.51	0.26	5.50	5.78
	120	6.27	0.12	0.55	0.29	5.34	5.79
P200910	55	6.87	0.15	0.48	0.33	5.34	5.71
	70	6.80	0.11	0.50	0.34	5.29	5.71
	70	4.93	-0.011	--	--	--	--
	90	6.71	0.14	0.49	0.37	5.34	5.73
	120	6.50	0.13	0.50	0.43	5.28	5.69

<sup>a</sup>  $\mu$  = specific growth rate

<sup>b</sup>  $Y_p$  = propionic acid yield

<sup>c</sup>  $Y_a$  = acetic acid yield

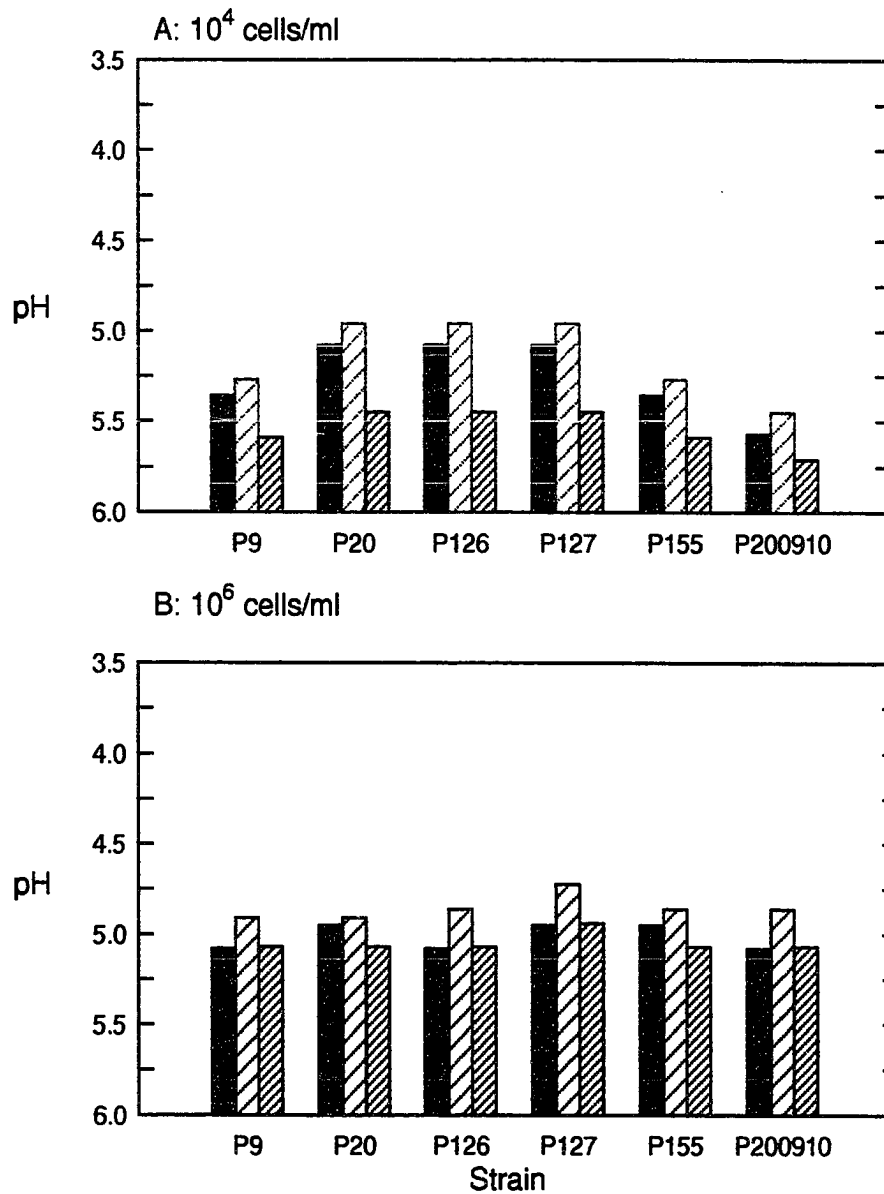





Figure 1: Comparison of minimum pH values at which growth was observed in broth acidified with lactic, hydrochloric, or propionic acid. Inoculum was  $10^4$  [A] or  $10^6$  [B] cells  $\text{ml}^{-1}$ . Inoculum level and nature of acidulant (inorganic vs organic) were found to be significant ( $P < 0.0001$ ). Symbols:  lactic acid;  hydrochloric acid;  propionic acid.

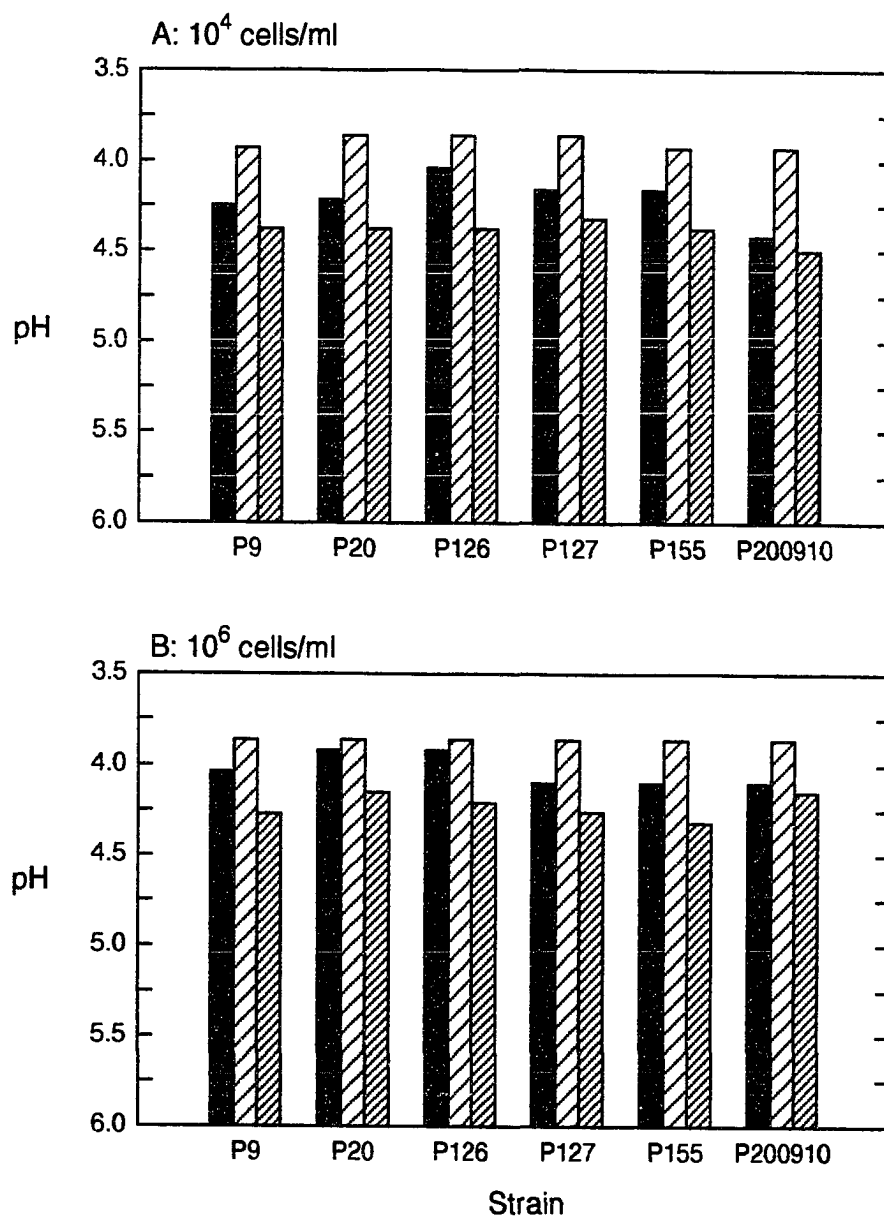
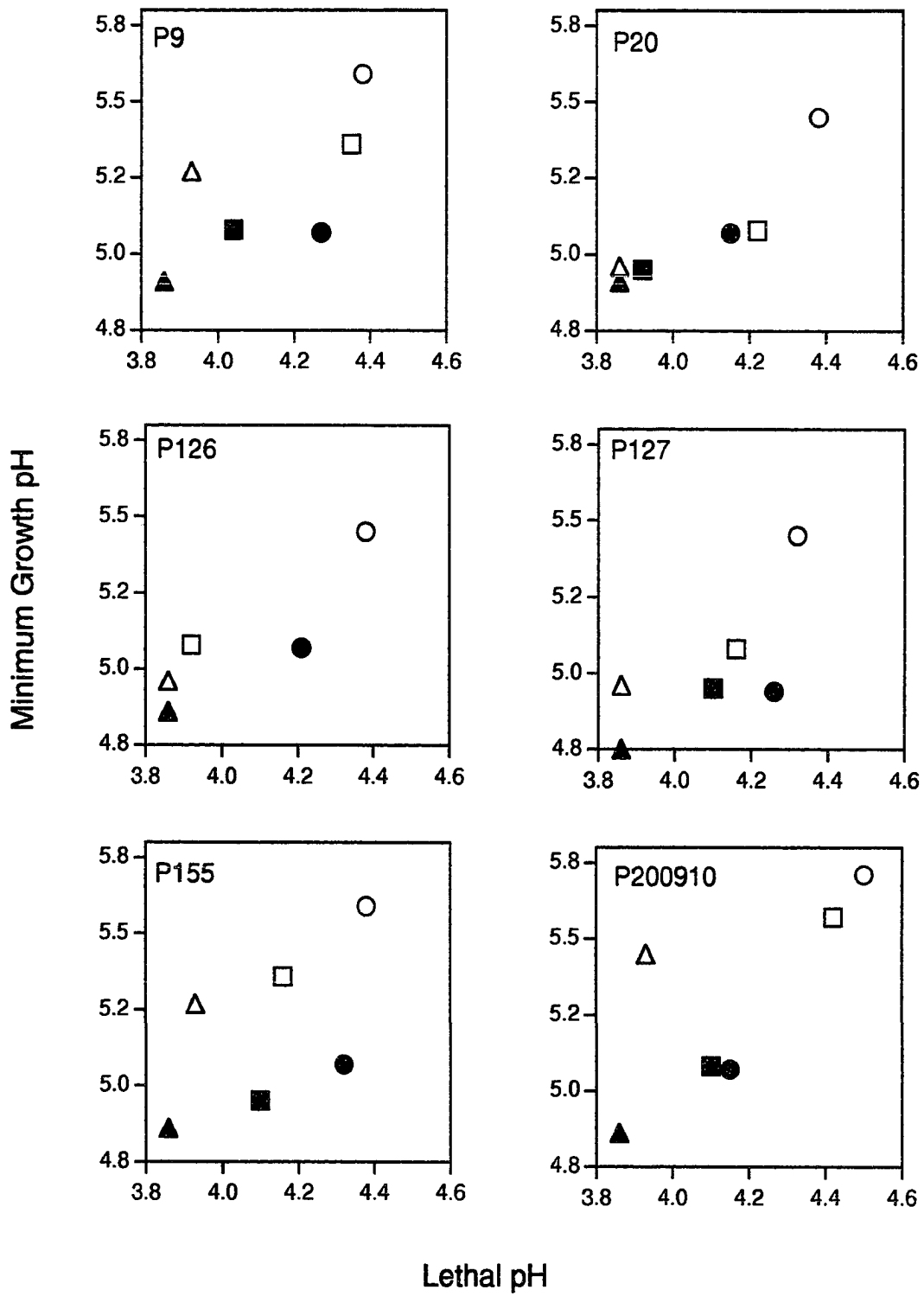


Figure 2: Comparison of lethal pH values in broth acidified with lactic, hydrochloric, or propionic acid. Inoculum was  $10^4$  [A] or  $10^6$  [B] cells  $\text{ml}^{-1}$ . Inoculum level and nature of acidulant (inorganic vs organic) were found to be significant ( $P < 0.0001$ ). Symbols:  lactic acid;  hydrochloric acid;  propionic acid.

Figure 3: Comparison of minimum growth and lethal pH values for strains of *Propionibacterium* inoculated at  $10^4$  cells  $\text{ml}^{-1}$  and  $10^6$  cells  $\text{ml}^{-1}$  in broth acidified with lactic, hydrochloric, or propionic acid. Symbols:  $\square$ , lactic acid at  $10^4$  cells  $\text{ml}^{-1}$ ;  $\Delta$ , HCl at  $10^4$  cells  $\text{ml}^{-1}$ ;  $\circ$ , propionic acid at  $10^4$  cells  $\text{ml}^{-1}$ ;  $\blacksquare$ , lactic acid at  $10^6$  cells  $\text{ml}^{-1}$ ;  $\blacktriangle$ , HCl at  $10^6$  cells  $\text{ml}^{-1}$ ; and  $\bullet$ , propionic acid at  $10^6$  cells  $\text{ml}^{-1}$ .





**THE EFFECT OF LOW PH, ACID, AND GROWTH ON THE  
PROTON MOTIVE FORCE OF *PROPIONIBACTERIUM*  
*THOENII* P127 IN BATCH CULTURE**

A paper prepared for submission to Applied and Environmental Microbiology

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**Abstract**

The transmembrane electrical potential ( $\Delta\Psi$ ) and pH gradient ( $\Delta\text{pH}$ ) of cultures of *P. thoenii* P127 were measured during batch growth started at pH 7 or pH 5 without pH control. The cells generated a  $\Delta p$  during growth, reaching a maximum of -169 mV when inoculated at pH 7 and -201 mV when inoculated at pH 5. The internal pH of cells of both cultures decreased as the external pH decreased. When grown at controlled pH, cells reached a maximum  $\Delta p$  of -173 mV at 144 h at pH 7, and a maximum  $\Delta p$  of -181 mV at 240 h at pH 5. The  $\Delta p$  at pH 7 was comprised of only  $\Delta\Psi$ , i.e. the internal pH remained constant at pH 7. At pH 5, however, the  $\Delta p$  consisted of both  $\Delta\text{pH}$  and  $\Delta\Psi$ . The internal pH decreased slowly throughout the fermentation from pH 6.06 at 48 h until it reached pH 5.03 at 930 h, at which time the  $\Delta\text{pH}$  collapsed; the critical internal pH threshold appeared to be pH 5.8. At pH 5 the specific growth rate ( $\mu$ ) was slower than at pH 7 ( $0.037\text{ h}^{-1}$  vs.  $0.13\text{ h}^{-1}$ ); ATP concentrations at mid-log phase were lower (2.05

mM vs. 3.28 mM); and propionic and acetic acid yields from lactate were lower (0.58 and 0.23 vs. 0.60 and 0.28 for propionic and acetic acid, respectively). The external pH did not affect the overall  $\Delta p$  values, but did affect the individual components of  $\Delta p$ , i.e.  $\Delta \Psi$  and  $\Delta pH$ .

## Introduction

Propionibacteria are fermentative organisms that produce propionic and acetic acids from the metabolism of lactate. Although they are primarily used in the dairy industry as starter cultures for Swiss-type cheeses (10), propionibacteria are also used industrially as silage inoculum (6, 25), as a probiotic agent (18) and for production of vitamin B<sub>12</sub> and propionic acid (22). However, currently commercial production of propionic acid is entirely by petrochemical routes. Fermentation has not been used industrially because propionibacteria grow slowly and produce organic acids in relatively low concentrations, and the costs of separation and concentration of the acids make recovery difficult and expensive (22). If higher concentrations of propionic acid could be obtained, the economics of the fermentation could be improved.

A major factor that limits the production of propionic acid during fermentation is end-product inhibition by the acid (9). Organic acids inhibit microorganisms by entering the cell in the undissociated form and then dissociating within the cell; this causes acidification of the cytoplasm and collapse of the proton motive force ( $\Delta p$ ) (9, 14, 21, 33). Some researchers believe that

organic acids act as “uncouplers” because they allow protons to enter the cell from the medium. The membrane-bound ATPase must function to counteract the futile cycle of protons through the membrane and thus the cell becomes deenergized (1, 2, 14, 19) . Other researchers believe that inhibition is due to anion accumulation and that the intracellular release of protons by organic acids is overemphasized (4, 30, 31, 32, 33). Russell (4, 33) stated that the critical aspect of a particular bacterium’s pH sensitivity may vary, but that organic acid toxicity was more closely related to intracellular pH regulation and anion accumulation than to “uncoupling” per se.

Little is known about the physiological response of propionibacteria to low pH. Hsu and Yang (11) found that the optimum pH for cell growth of *P. acidipropionici* was from pH 6.0 to 7.1 and that the growth rate decreased at acidic pH values. However, propionic acid yields were higher at low pH because the cells diverted less energy to production of succinate and pyruvate.

The objectives of this study were to determine effect of low pH, growth, and acid production on the proton motive force of *P. thoenii* P127. Previous attempts to isolate acid- and/or pH-tolerant variants of *Propionibacterium* led to the discovery that propionibacteria are very sensitive to low pH and cannot grow below pH 5.0 (26). However, they are tolerant to relatively high concentrations of lactic acid at neutral pH (up to 3.5%). To our knowledge, this is the first report of the measurement of the proton motive force of propionibacteria.

## **Materials and Methods**

### **Organism and culture conditions**

*Propionibacterium thoenii* P127 was obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Growth medium was sodium lactate broth (NLB) which contained 1% trypticase soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA) and 0.6% Tween 80 (Fisher). For experiments conducted at lowered pH values, NLB was acidified to the desired pH with lactic acid. Inocula used in all experiments were grown in NLB incubated at 32°C for 24 h followed by at least two sequential transfers of 1% inocula into NLB.

Cells for transmission electron microscopic analysis were prepared by growing P127 in 250-ml screw-capped Erlenmyer flasks containing 100 ml of NLB at pH 7 or pH 5. The cultures were incubated at 32°C until mid-logarithmic growth (24 h for pH 7 and 72 h for pH 5). To avoid a low final pH because of acid production, the pH 7 culture was centrifuged (9,000 x *g* for 15 min at 4°C) and resuspended in fresh pH 7 NLB at 20 h. The final pH was 6.2.

### **Fermentation conditions**

Batch fermentations without pH control were performed in a 2-L Erlenmeyer flask which contained 1.8 L NLB at pH 7 (55 mM lactate) or pH 5 (70

mM lactate). Samples of approximately 50 ml were taken every 12 to 24 h for analyses. To avoid large changes in head space that would result from sampling a single flask, six replicate fermentations were conducted and sampled at different times. Each sample time was duplicated; all fermentations were sampled at 24 h.

Batch fermentations with pH control were conducted in a 2-L bench-top fermenter (Biostat M, B. Braun Biotech. Inc., Allentown, PA) with a working volume of 1.8 L. The temperature was controlled at 32°C, the agitation was 150 rpm, and the pH was controlled at pH 7 or pH 5  $\pm$  0.1 pH unit with 3 N NaOH. Samples were taken at timed intervals for analyses. At 18, 24, and 36 h, 60-ml samples were taken from the fermenter and replaced with 60 ml of fresh NLB. At other sample times, smaller volumes (approximately 25 ml) were taken and were not replaced with fresh NLB.

### **Proton motive force determination**

Estimation of the proton gradient ( $\Delta$ pH) and the electrical potential ( $\Delta\psi$ ) was based on distribution of [ $^{14}$ C]salicylic acid and [ $^3$ H]tetraphenylphosphonium bromide (TPP $^+$ ), respectively (13, 21, 29). The accumulation of salicylate and TPP $^+$  was determined by the silicon oil centrifugation technique derived from Rottenberg (29) and Tseng *et al.* (36). Briefly, the method was as follows.

Samples were centrifuged at 9000  $\times g$  for 15 min at 4°C and resuspended in the spent NLB to an absorbance of 1.0 at 600 nm. Aliquots of 1 ml were placed into microcentrifuge tubes and [ $^{14}$ C]salicylate (2.5  $\mu$ M, 0.125  $\mu$ Ci; Dupont NEN, Boston, MA), [ $^{14}$ C]PEG (3.9  $\mu$ M, 0.25  $\mu$ Ci; Amersham Corp., Arlington Heights, IL), [ $^3$ H]tetraphenylphosphonium bromide (2.25  $\mu$ M, 0.5  $\mu$ Ci; Amersham), or  $^3\text{H}_2\text{O}$

(1.25  $\mu\text{Ci}$ ; Amersham) was added to duplicate tubes. After a 15-min incubation at room temperature, 0.5 ml of silicone fluid, density = 1.025 g ml<sup>-1</sup> (50:50 mixture of Fluid 550 and Fluid 556; Dow Corning Corp., Midland, MI) was layered on top of each sample and the tubes were centrifuged at 25°C at 13,000 x *g* for 3 min. A 100- $\mu\text{l}$  sample of supernatant was pipetted into a scintillation vial (Research Products International Corp., Mount Prospect, IL), 4 ml of 30% Scintiverse (Fisher) were added, and the vial was thoroughly mixed with a vortex mixer. The remaining supernatant was carefully removed with a pipet and cotton swab, and the oil was drained from the tubes. The bottom of the tube, containing the pellet, was cut and allowed to fall directly into a scintillation vial that contained 1 ml of 0.3N perchloric acid. The vial was thoroughly mixed by vortex to dissolve the pellet, 4 ml of 30% Scintiverse were added, and the vial was mixed again. Radioactivity was measured with a Packard Liquid Scintillation Analyzer Model 1900 TR (Packard, Meriden, CT) for 5 min in the DPM mode. Corrections for nonspecific binding of the probes were based on the extent of probe accumulation in cells treated with 20  $\mu\text{M}$  valinomycin (Sigma, St. Louis, MO) and 5  $\mu\text{M}$  nigericin (Sigma) for 60 min.

### Calculations

The internal volume of the cells was calculated as outlined by Kashket (12) from the difference between the total (<sup>3</sup>H<sub>2</sub>O permeable) and extracellular (<sup>14</sup>C-PEG-nonpermeable) volume of pellets. The internal volume of the cells in the pH 7 fermentations was calculated as 0.96 +/- 0.13  $\mu\text{l mg}^{-1}$  dry weight; for pH 5 fermentations the internal volume was calculated as 0.51 +/- 0.068  $\mu\text{l mg}^{-1}$  dry

weight. The calculated internal volumes were the averages of at least six different measurements determined at several different phases of culture growth. Although the volumes decreased slightly as the culture went into stationary phase, these differences were not significant and thus one value was used in the calculations of  $\Delta\text{pH}$  and  $\Delta\Psi$ .

The  $\Delta\text{pH}$  was calculated from the distribution ratio of [ $^{14}\text{C}$ ]salicylic acid by using the Henderson-Hasselbalch equation (13, 21, 29):

$$\Delta\text{pH} = \log \frac{[\text{salicylic acid}]_{\text{in}}}{[\text{salicylic acid}]_{\text{out}}}$$

The  $[\text{salicylic acid}]_{\text{in}}$  was determined from the DPM in the pellets divided by the cell volume, while the  $[\text{salicylic acid}]_{\text{out}}$  was determined from the DPM in the supernatant (21, 29). The  $\Delta\Psi$  was calculated in a similar manner (13, 21, 29):

$$\Delta\Psi = -Z \log \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}}$$

Where  $z = 59 \text{ mV}$  at  $25^\circ\text{C}$ . The proton motive force was calculated as (13, 21, 29):

$$\Delta p = \Delta\Psi - Z\Delta\text{pH}$$

In this paper, negative signs will be used for  $\Delta\Psi$  (i.e. negative inside) and for  $\Delta p$  (negative and alkaline inside compared to the external medium). In discussions comparing the  $\Delta\Psi$  and  $\Delta\text{pH}$ ,  $Z\Delta\text{pH}$  is used because multiplication by  $z$  converts the  $\Delta\text{pH}$  to the same units (millivolts) as  $\Delta\Psi$ .

### **Transmission Electron Microscopy (TEM)**

Cell preparation and electron microscopy were performed at the Bessey Microscopy Facility at Iowa State University. Mid-logarithmic cells were fixed for

1 h at room temperature in 3% glutaraldehyde (Electron Microscopy Sciences (EMS), Fort Washington, PA) and 2% paraformaldehyde (EMS) in phosphate buffer adjusted to either pH 7 or pH 5. Cells were washed three times in phosphate buffer adjusted to pH 7 or pH 5 and post fixed with 1% osmium tetroxide (EMS) for 30 min. The cells were washed and *en bloc* stained with 2% aqueous uranyl acetate overnight at 4°C, washed, and dehydrated in a graded ethanol series into pure acetone. The cells were embedded in epon resin (EMS) and thin sectioned (50 to 60 nm) on a Reichert Ultracut S ultramicrotome (Leica, Vienna, Austria). Thin sections were placed on copper grids and stained with 3% methanolic uranyl acetate Reynolds lead stain (27) and viewed on the JEOL 1200EXII transmission electron microscope (JOEL, Peabody, MA) at 30,000 x magnification.

### **Analysis of products**

Lactate, propionate, and acetate were separated by a high-performance liquid chromatography (HPLC) system equipped with a model 501 pump (Waters, Division of Millipore, Milford, MA) and an HPX-87H column (BioRad, Richmond, CA) operated at 65°C with 0.012 N H<sub>2</sub>SO<sub>4</sub> (pH 2.0) as the mobile phase at a flow rate of 0.8 ml min<sup>-1</sup> and an injection volume of 20 µl. Peaks were detected with a Waters differential refractometer (model R401). Samples for analysis were centrifuged to remove the cells, filtered through 0.2 µm filters, and stored at -20°C before analysis. A Maxima 820 software program (Waters) was used to analyze the data and plot the chromatograms. Product concentrations were calculated by



comparing the peak areas with those of external standards (Aldrich Chemical Co., Milwaukee, WI).

For ATP determination, samples from the fermenter were prepared according to the method of Strobel and Russell (35). Samples consisted of cells resuspended in spent medium to an absorbance of 2.0 at 600 nm (4.36 mg dry weight ml<sup>-1</sup>). The ATP was measured by the firefly luciferase method (17). The supernatant was diluted 100-fold with 40 mM Tris buffer (Fisher) that contained 2 mM EDTA (Fisher), 10 mM MgCl<sub>2</sub> (Fisher) and 0.1% bovine serum albumin (Sigma) (pH 7.75). The luciferase reaction was initiated by mixing 100 µl of the diluted sample with 100 µl of luciferine-luciferase assay mix according to the recommendations of the supplier (Sigma). Light intensity was measured with a Packard Model 1900 TR Liquid Scintillation Analyzer using ATP as the standard (20).

An analysis of variance was performed using the General Linear Model of SAS (34). Differences with  $P < 0.01$  were considered significant.

## **Results and Discussion**

### **Fermentations without pH control**

No information concerning the proton motive force and internal pH was previously available for propionibacteria and it was not known how these parameters would respond to growth and acidic pH. Previous studies determined that *P. thoenii* P127 was sensitive to low pH and was not able to grow below pH 5

(26). Measuring the cellular response at pH 7 and pH 5 may indicate the critical aspect(s) of low pH inhibition and tolerance. To determine how culture growth and acidification of the medium affected the cellular proton motive force and internal pH of this strain, P127 was grown at starting pH values of pH 7 and pH 5 in batch culture without pH control (Figure 1). The  $\Delta p$  of the culture inoculated at pH 7 increased throughout exponential growth from -118 mV at 12 h to -134 mV at 36 h and reached a maximum at 48 h of -169 mV. The  $\Delta p$  then decreased to a minimum of -53 mV at 192 h. For cultures inoculated at pH 5, the  $\Delta p$  increased from -99 mV at 48 h to the maximum value of -201 at 144 h. There was a decrease in  $\Delta p$  at 192 h to -137 mV.

As previously observed in bacteria, the relative contribution of  $\Delta \Psi$  and  $\Delta pH$  to  $\Delta p$  are dependent on the pH of the medium (2, 12). Throughout the fermentation, the  $\Delta p$  of the culture inoculated at pH 7 consisted of a large  $\Delta \Psi$  and a small  $\Delta pH$ . While for the cultures inoculated at pH 5, the  $\Delta pH$  was the major component of the  $\Delta p$  for the first 72 h. After this time, the  $\Delta \Psi$  was the major component. The  $\Delta pH$  of the pH 5 culture was larger than that of the pH 7 culture at all sample times. This resulted in a larger  $\Delta p$  for the culture inoculated at pH 5.

During logarithmic growth, the internal pH (external pH +  $\Delta pH$ ) of the pH 7 culture without pH control decreased as the external pH decreased, until the internal pH reached approximately 6.4, (external pH was approximately 5.5) (Figure 2). The internal pH then remained relatively constant until the  $\Delta pH$  collapsed at 192 h. The culture started at pH 5 maintained a relatively constant internal pH of approximately 6.0 throughout the growth phase while the external pH decreased only slightly to 4.8. The  $\Delta pH$  began to decrease at 192 h, which

resulted in an internal pH of 5.8. The decrease in internal pH with the external pH is consistent with observations made with other fermentative microorganisms including *Clostridium thermoaceticum* (1), *Streptococcus cremoris* (3), *Sarcina ventriculi* (8), and various lactic acid bacteria (4, 14, 19).

### **Fermentations with pH control**

To measure the response of P127 to acid production at neutral and low pH, the strain was grown in batch culture at pH 7 and 5 with pH control (Figures 3 and 4). As previously observed in propionibacteria (11, 16), the specific growth rate was significantly slower at the lower pH;  $\mu$  was  $0.13 \text{ h}^{-1}$  at pH 7 and  $0.037 \text{ h}^{-1}$  at pH 5. However, the pH 5 cultures grew to a higher total cell count:  $2.1 \times 10^9 \text{ cfu ml}^{-1}$  versus  $9.1 \times 10^8 \text{ cfu ml}^{-1}$  at pH 7.

At pH 7, acid production followed a growth-associated pattern. Lactate was depleted by 72 h; propionic acid concentration was highest ( $6.17 \text{ g l}^{-1}$ ) at 192 h while acetic acid was highest ( $3.07 \text{ g l}^{-1}$ ) at 144 h. Acid production at pH 5 was significantly slower; propionic and acetic acid concentrations were highest at 666 h ( $6.21 \text{ g l}^{-1}$  and  $3.36 \text{ g l}^{-1}$ , respectively) while the culture was in death phase. Lactate consumption rate decreased at 240 h; lactate was not depleted until 552 h. This is consistent with previous observations that when *P. acidipropionici* was grown at pH 5, acid production and lactose consumption continued after cells had entered stationary phase (11). Propionic and acetic acid yields (gram acid produced per gram lactate consumed) were slightly higher at pH 7: 0.60 and 0.28, respectively and 0.58 and 0.23 for pH 5, respectively.

The ATP concentration in the pH 7 culture decreased with lactate consumption and propionic and acetic acid production during logarithmic growth, but increased at the beginning of stationary phase (from 1.43 mM at 72 h to 2.97 mM at 96 h). The ATP concentration remained elevated through 144 h and then decreased dramatically at 240 h to 0.46 mM. This transient increase occurred in all three pH 7 fermentations conducted and may have been due to metabolism of amino acids or other growth factors in the medium. Strains of propionibacteria have been found to utilize amino acids, especially alanine, aspartate, glycine, and serine (5, 15). Alanine was not metabolized until after lactate had been depleted (5). This shift in metabolism may have supplied additional ATP to the cells.

At pH 5, the ATP concentration decreased along with lactate consumption and propionic and acetic acid production from 2.05 mM at 72 h to 0.38 mM at 552 h, and increased only slightly at 168 h. For both pH 7 and 5 fermentations, the cells entered death phase when ATP levels were below approximately 0.5 mM. Previous researchers found that in the presence of propionic and lactic acids, *L. helveticus* increased its H<sup>+</sup>-ATPase activity (24). This enzyme utilized glycolytically generated ATP to expel protons accumulated by the cell as a result of lactic acid production and/or exposure to propionic acid. In addition, growth rate and biomass production decreased when metabolism could not supply ATP required for proton extrusion (23).

During log-phase growth at pH 7, the proton motive force potential increased from -87 mV at 18 h to -137 mV at 36 h and reached a maximum at the beginning of stationary phase (144 h) of -173 mV; after this time the  $\Delta p$  began to be depleted.

However, with extended incubation the  $\Delta p$  began to increase again, from -119 mV at 480 h to -158 mV at 1500 h. There was also an increase in acetic acid concentration and in viable counts, from 3.04 g l<sup>-1</sup> and 2.9 x 10<sup>7</sup> cfu ml<sup>-1</sup> at 480 h to 3.43 g l<sup>-1</sup> and 8.1 x 10<sup>7</sup> cfu ml<sup>-1</sup> at 1500 h. These data may suggest that the culture had undergone some metabolic change which may have induced the production of new enzymes. An SDS-PAGE of intracellular proteins obtained from cells at 1500 h and from logarithmically growing cells contained an additional band in cells at 1500 h at approximately 25 kDa (Appendix B). The culture may have synthesized enzymes necessary for the utilization of the amino acids remaining in the medium. The protein might also correspond to a protein synthesized during stationary phase.

At pH 5, the proton motive force increased from -67 mV at 48 h to -113 mV at 120 h; at the beginning of stationary phase (240 h) it reached a maximum of -181 mV. Throughout stationary phase, the  $\Delta p$  steadily decreased to a minimum at 810 h (-40 mV) and was collapsed at 930 h.

Throughout the entire pH 7 batch fermentation, the  $\Delta p$  consisted entirely of the  $\Delta \Psi$ , i.e. there was no measurable pH gradient. The internal pH remained constant at approximately 7.0. Others have reported that *Streptococcus cremoris* also had no measurable  $\Delta pH$  when grown in batch culture at pH 7; the  $\Delta pH$  did not develop until the external pH decreased with acid production (12).

In contrast, for the first 96 h of the pH 5 fermentation the  $\Delta p$  was comprised of only the  $\Delta pH$  component, i.e. there was no measurable  $\Delta \Psi$ . At all sample times, the  $\Delta \Psi$  of the pH 5 culture was lower than that of the pH 7 culture. At mid log-phase, the  $\Delta \Psi$  of the pH 7 culture was -132 mV, while at pH 5 the  $\Delta \Psi$  was

zero. In contrast, the  $\Delta\text{pH}$  of the pH 5 culture was -63 mV and zero at pH 5.

These results are consistent with previous observations that the activity of proton pumps is constrained by the generation of the proton motive force; to generate a pH gradient, excessive membrane potential must be dissipated (2).

The internal pH of the pH 5 culture remained relatively constant at the beginning of the fermentation (6.06 to 5.93), but began to decrease gradually after 96 h (Table 1). The ability of a culture to tolerate acidic conditions has been attributed to its ability to lower its internal pH with the pH of the medium in the presence of weak organic acids (4, 30, 31, 32, 33). Weak organic acids will diffuse across the membrane in accordance with the pH gradient and dissociate at the more alkaline internal pH. The impermeable anion will accumulate based on the Henderson-Hasselbalch equation (33). During fermentation, P127 decreased its internal pH with acid production and thus the propionic and acetic acid gradient across the membrane decreased (Table 2). If P127 had maintained a constant internal pH, the gradient would have remained the same and would have resulted in higher internal concentrations of propionic and acetic acids.

The  $\Delta\text{pH}$  decreased from 0.80 to 0.68 pH unit from 336 to 456 h, which resulted in a decrease in internal pH from 5.8 to 5.6. At the same time the cells entered death phase and the rate of lactate consumption decreased, even though the lactate had not been depleted. This may suggest that an internal pH of 5.8 may be the critical pH threshold for strain P127. Most internal enzymes have a narrow range in which they are active (2, 14); pH 5.8 may be the lower limit for enzymes in this strain. Similar values were measured for *Leuconostoc mesenteroides* (19). McDonald *et al.* (19) demonstrated that growth of

*Leuconostoc mesenteroides* terminated when its internal pH reached 5.4 to 5.7 at an external pH of 4.0.

Although the external propionic and acetic acid concentrations were similar for both pH 7 and pH 5 fermentations, at pH 7 the acids were in the dissociated form and thus could not penetrate the cell membrane. In contrast, at pH 5 about half of the total amount of the acids was in the undissociated form (Table 2). This form could re-enter the cell and acidify the cytoplasm (4, 14, 19, 33). The lowering of the internal pH after 96 h may result from the increase in undissociated acetic and propionic acid concentrations.

The ATP concentrations for the pH 5 culture were significantly lower than for the pH 7 culture; this may be due to the reverse action of the  $H^+$ -ATPase which consumes ATP to expel protons in order to maintain an alkaline internal pH with respect to the medium (1, 2, 21). We had expected the pH 5 culture to reach lower total cell numbers than the pH 7 culture because less energy was available at the lower pH for biosynthesis of macromolecules and cell division. The culture at pH 5 also might have rapidly consumed the lactate in order to keep up with the demand for ATP. Neither of these anticipated results was observed, however. Results similar to ours were observed with *L. lactis* by Cook and Russell (4). They found that *L. lactis* decreased its intracellular pH, glycolytic rate, and intracellular ATP concentration when the external pH became acidic, and concluded that energy availability for growth was a key factor in the pH sensitivity of this organism. Thus, it appears that energy availability may also play an important role in pH sensitivity of P127.

During the pH 5 fermentation, a base pump malfunction caused the pH of the medium to increase to pH 5.23 at 168 h. The  $\Delta$ pH measured at this time was significantly lower (0.34 pH unit) than the  $\Delta$ pH measured at the previous (120 h) and subsequent (240 h) sample times (0.84 and 0.80 pH unit, respectively) (Table 1). The decrease in  $\Delta$ pH at 168 h indicated both a lower internal pH (5.57) and a lower acid gradient across the membrane (1.8 for propionic acid and 1.9 for acetic acid). The lower acid gradient would result in less acid transversing the membrane and accumulating inside. There was also an increase in ATP from 0.88 mM at 120 h to 1.18 mM at 168 h (Figure 4). This transient increase may have occurred because there was a lower demand for ATP due to less accumulation of protons with incoming acid.

### **Effect of acidic pH on cell wall and membrane**

To determine the effect of growth at low pH on the cell wall and membrane of strain P127, cells grown at pH 7 and pH 5 were examined by transmission electron microscopy. The electron micrographs show that cells grown at pH 7 (Figure 5A and 5B) had a more uniformly dense cell wall than did cells grown at pH 5 (Figure 5C and 5D). The walls of cells grown at pH 5 appeared to be thicker than were those of cells grown at pH 7. They also seemed to lack definitive structure (i.e. no dark band at the outer and inner edge of the cell wall and no indication of the bilayer of the membrane).

These differences might be due to changes occurring at pH 5 in the murein structure, fatty acid composition of the membranes, composition or amount of extrapolsaccharides produced, or possibly in uptake of the microscopy stains



(uranyl acetate and lead citrate). Teichoic acids have been found to play a role in the expansion and contraction of cell walls that occurred with alterations in pH and ionic strength (28). There may also have been a difference in the amount or composition of the teichoic acids found in the cell walls at the different pH values.

### **Comparison of $\Delta\Psi$ and $\Delta p$ values**

To determine whether it was the actual value of the external pH or the presence or absence of pH control that contributed the most to the variance of  $\Delta p$  and  $\Delta\Psi$  values, a comparison was made of these values during growth phase only of cultures grown with and without pH control. Because cultures grew more slowly at pH 5 than at pH 7, absorbances at different sample times were compared to the highest absorbance attained by that culture. The  $\Delta p$  and  $\Delta\Psi$  values were plotted against these standardized absorbance values. A linear regression was done separately for each pH and treatment (with and without pH control) using the General Linear Model of SAS (34). The statistically predicted lines are shown in Figure 6. The  $\Delta p$  values differed significantly between cultures with and without pH control, but did not significantly differ between pH 7 and pH 5. At mid-growth, the average  $\Delta p$  for cultures without pH control was 31% larger than the  $\Delta p$  of the pH-controlled cultures. This difference was due to larger  $\Delta pH$  values in both pH 7 and 5 fermentations without pH control.

In contrast, the  $\Delta\Psi$  values differed significantly between pH 7 and pH 5, but did not differ between the cultures with and without pH control. At mid-growth, the average  $\Delta\Psi$  for both cultures inoculated at pH 7 was 52% higher than the average

$\Delta\Psi$  for both pH 5 cultures. These results suggest that pH did not affect the overall  $\Delta p$  values, but did affect the individual components, i.e.  $\Delta\Psi$  and  $\Delta pH$ .

The  $\Delta p$  and  $\Delta\Psi$  of the cultures with the same initial pH responded to growth in the same manner, i.e. showed the same change in potential ( $\Delta p$  or  $\Delta\Psi$ ) with change in growth. This response (shown by the slopes of the lines) differed between cultures inoculated at pH 7 and pH 5, although this difference only comprised 8% of the total variance of the  $\Delta\Psi$  and  $\Delta p$  values.

It appeared that the  $\Delta p$  and  $\Delta\Psi$  were generated during growth at approximately the same rate in pH 7 and pH 5 cultures. Although pH significantly affected the specific growth rates and ATP concentrations of strain P127, pH did not affect the generation and magnitude of the  $\Delta p$ . However, pH affected the components that comprised the total  $\Delta p$ ; pH 5 cultures had larger  $\Delta pH$  and smaller  $\Delta\Psi$  components than did cultures at pH 7.

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Table 1: pH and acid gradients of strain P127 at controlled pH 5.0.

Time (h)	pH out <sup>a</sup>	$\Delta$ pH	pH in <sup>b</sup>
48	4.92	1.14	6.06
72	4.83	1.08	5.91
96	4.96	0.97	5.93
120	5.06	0.80	5.86
168	5.23	0.34	5.57
240	4.90	0.84	5.74
336	4.99	0.80	5.79
456	4.93	0.68	5.61
552	4.98	0.60	5.58
666	4.90	0.36	5.26
810	4.90	0.14	5.04
930	5.03	0	5.03

<sup>a</sup> external pH

<sup>b</sup> internal pH ( $\Delta$ pH + external pH)

Table 2: Concentration of dissociated and undissociated propionic and acetic acid and their gradients across the cell membrane in batch fermentation controlled at pH 5.

Time (h)	pH	Propionic acid (mM)				Acetic acid (mM)			
		Total <sup>a</sup>	A <sup>-b</sup>	HA <sup>b</sup>	In/out <sup>c</sup>	Total <sup>a</sup>	A <sup>-b</sup>	HA <sup>b</sup>	In/out <sup>c</sup>
48	4.92	24	13	11	7.8	0	0	0	0
72	4.83	33	16	17	6.3	0	0	0	0
96	4.96	39	22	17	5.6	0	0	0	0
120	5.06	49	30	19	4.2	31	21	10	4.5
168	5.23	66	46	20	1.8	42	31	11	1.9
240	4.90	72	37	35	4.1	45	26	19	4.4
336	4.99	75	43	32	4.0	49	30	19	4.3
456	4.93	79	42	37	3.0	53	31	22	3.2
552	4.98	83	47	36	2.7	57	34	23	2.9
666	4.90	84	43	41	1.7	59	33	26	1.8
810	4.90	80	41	39	1.2	56	31	25	1.2
930	5.03	79	47	32	1.0	58	37	21	1.0

<sup>a</sup>Total acid concentration as measured by HPLC

<sup>b</sup>A<sup>-</sup> = undissociated, HA = undissociated, both calculated from the Henderson-Hasselbalch equation, pK<sub>a</sub> propionic acid 4.87, pK<sub>a</sub> acetic acid 4.76

<sup>c</sup>Total acid gradient across the membrane calculated from the Henderson-Hasselbalch equation and the internal and external pH values.

Figure 1: Growth and the components of the proton motive force for cultures of *P. thoenii* P127 inoculated at pH 7 (a) and pH 5 (b) without pH control.  
Symbols: ▼, absorbance; ●,  $\Delta p$ ; □,  $\Delta \Psi$ ; and ◆,  $Z\Delta pH$ .



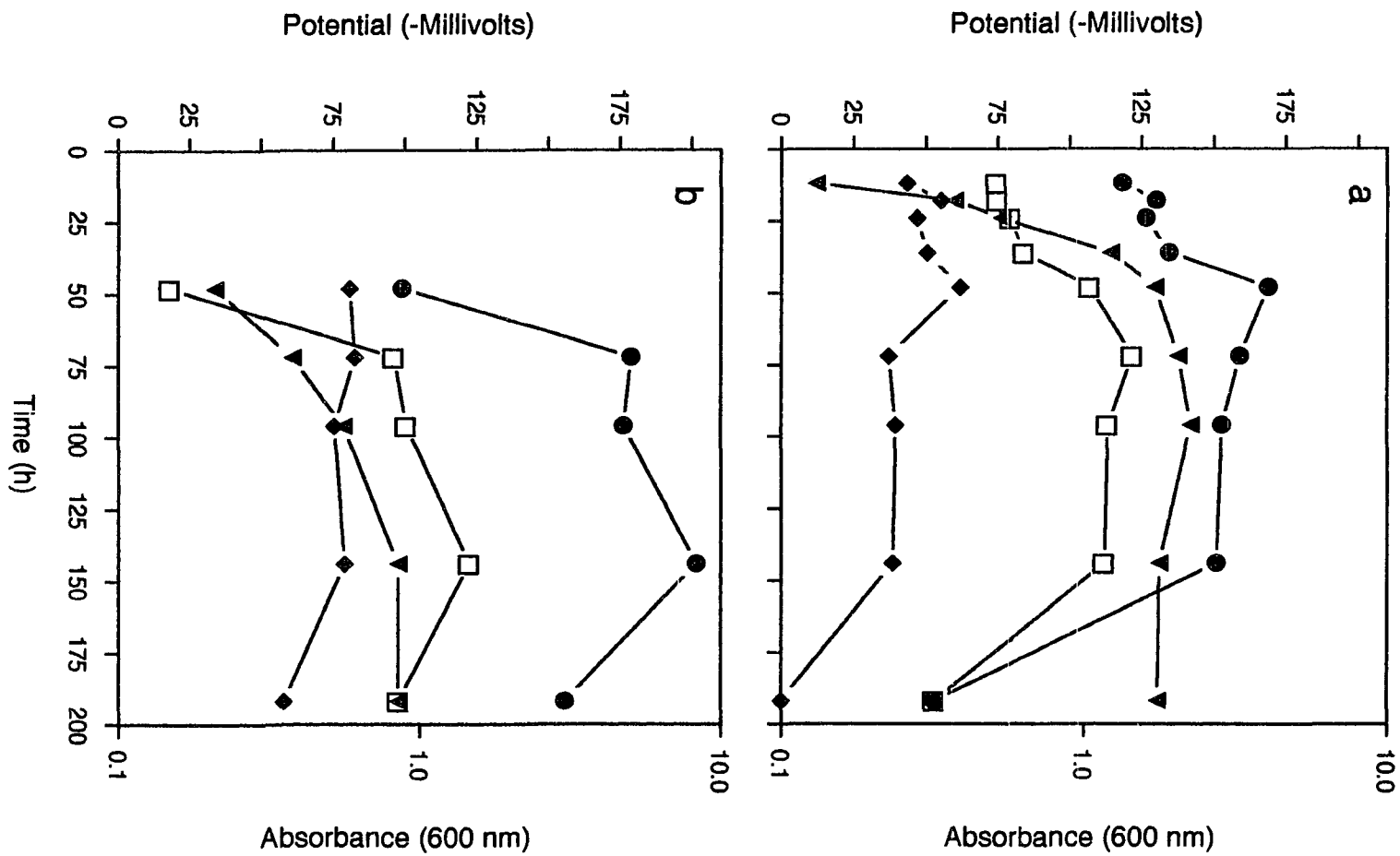


Figure 2: Internal and external pH values for P127 cultures inoculated at pH 7 and pH 5 without pH control. Symbols: ○, external pH at pH 7; □, internal pH at pH 7; ●, external pH at pH 5; and ■, internal pH at pH 5.

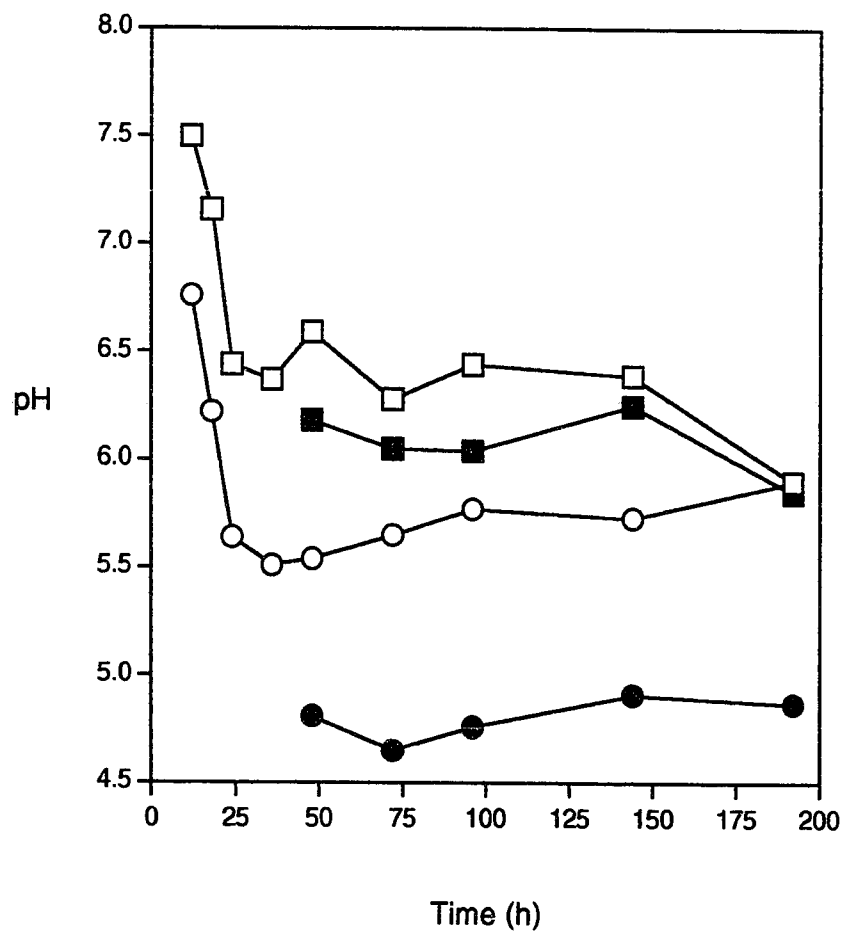


Figure 3: Growth, product formation, and culture characteristics of strain P127 at controlled pH 7.0. Symbols: ▼, absorbance; ※, viable counts; +, ATP; ▲, lactic acid; ○, propionic acid; Δ, acetic acid; and ●,  $\Delta p$  or  $\Delta \Psi$ .

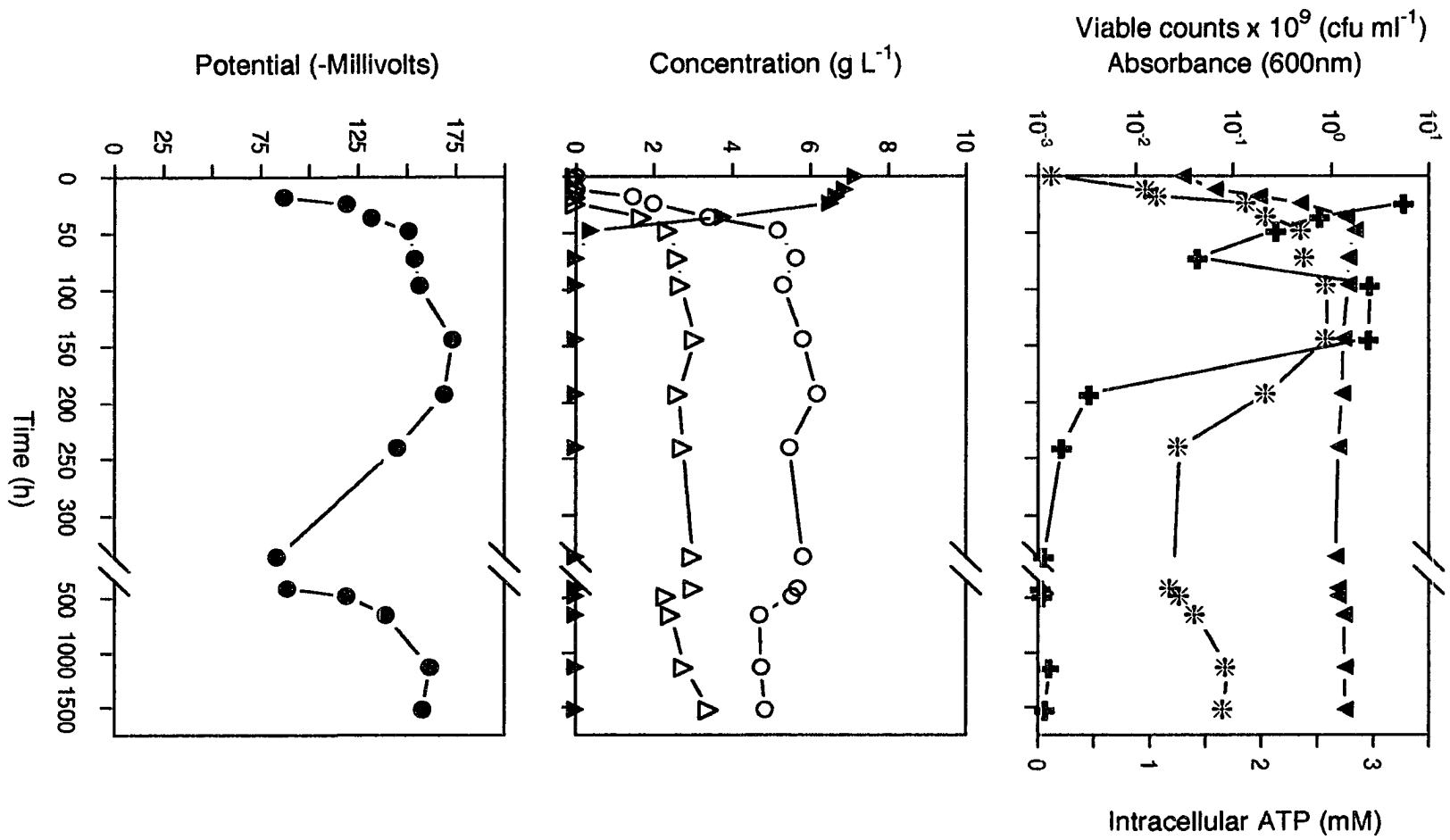


Figure 4: Growth, product formation, and culture characteristics of strain P127 at controlled pH 5.0. Symbols: ▼, absorbance; ※, viable counts; +, ATP; ▲, lactic acid; ○, propionic acid; Δ, acetic acid; ●,  $\Delta p$ ; □,  $\Delta \Psi$ ; and ◆,  $Z\Delta pH$ .

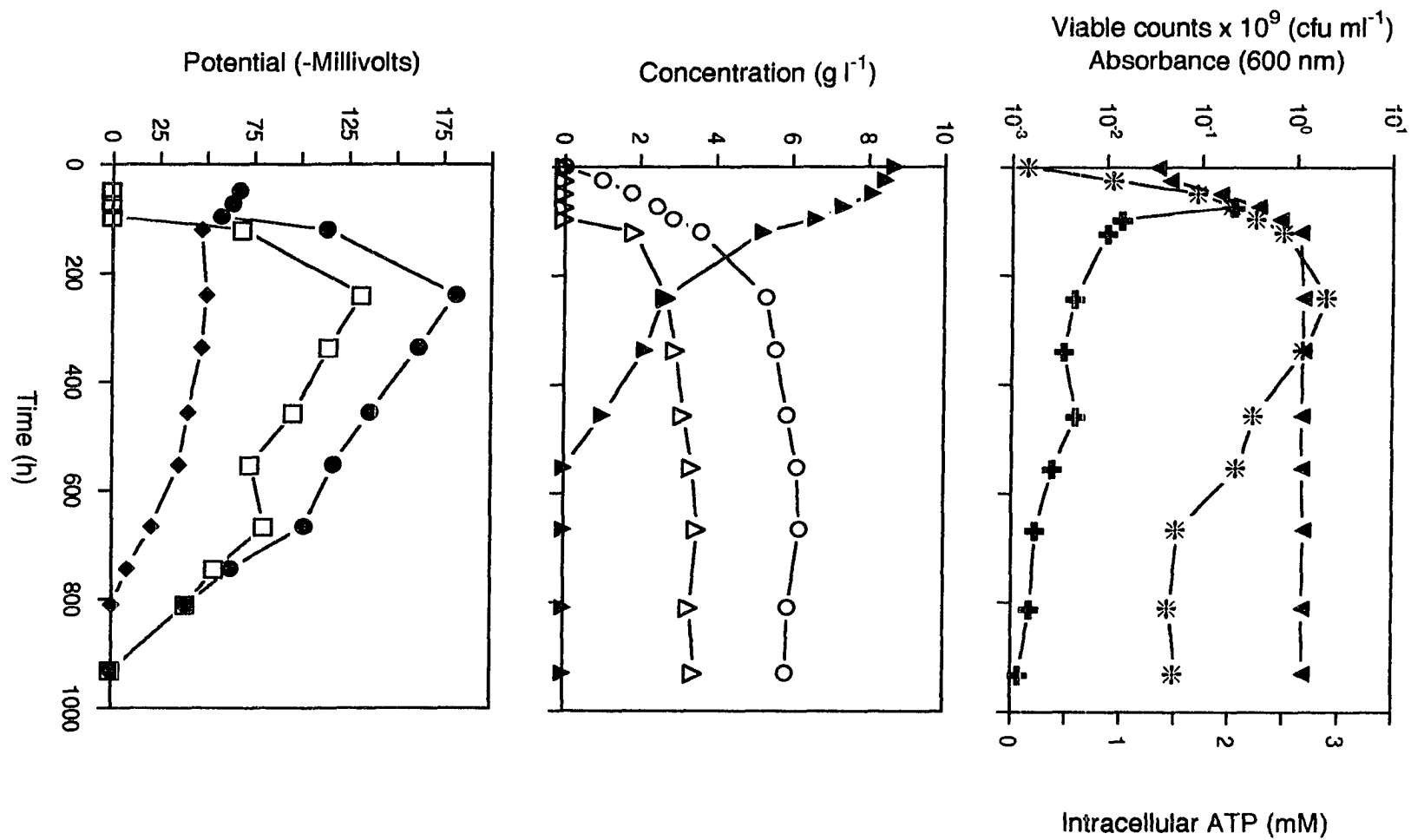


Figure 5: Transmission electron micrographs of thin sections of strain P127 grown at pH 7 (A and B) and pH 5 (C and D). Arrows, cell walls and membrane. Bars A and C, 300 nm; B and D, 150 nm.



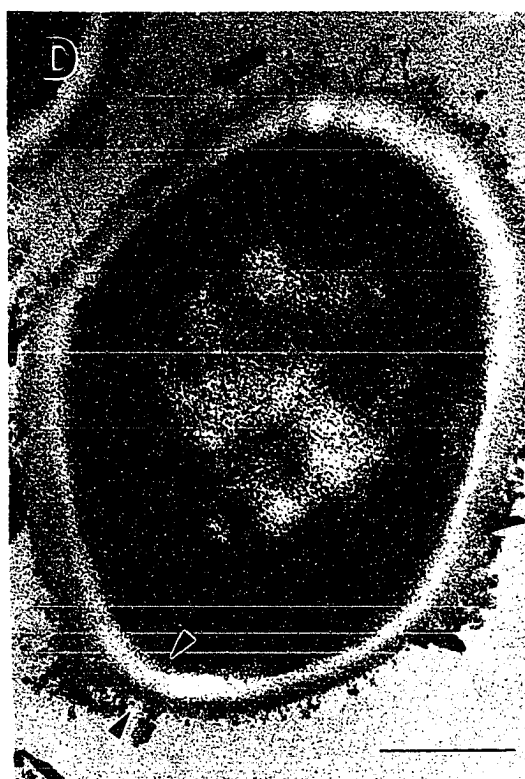
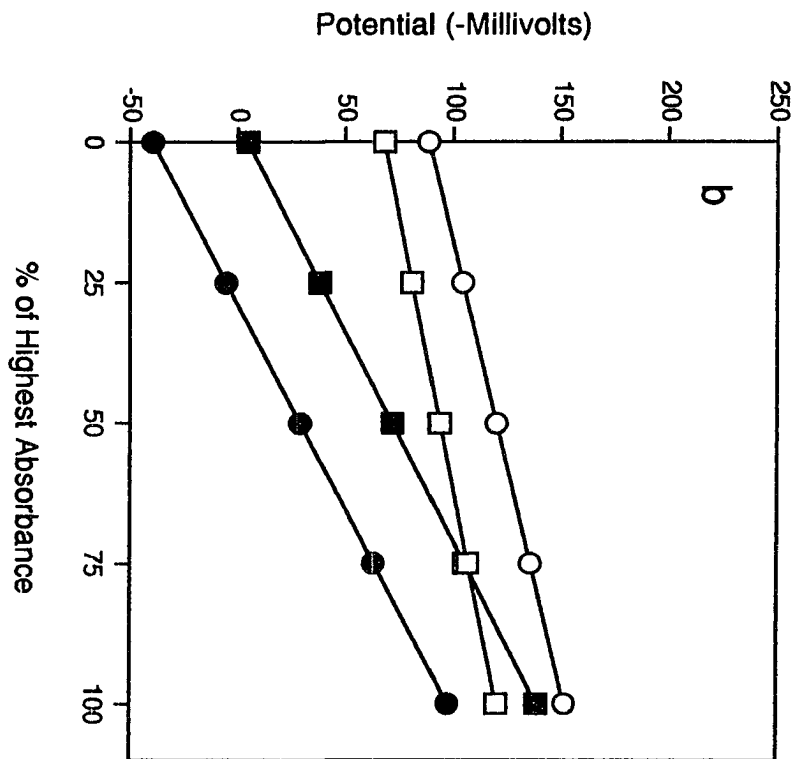
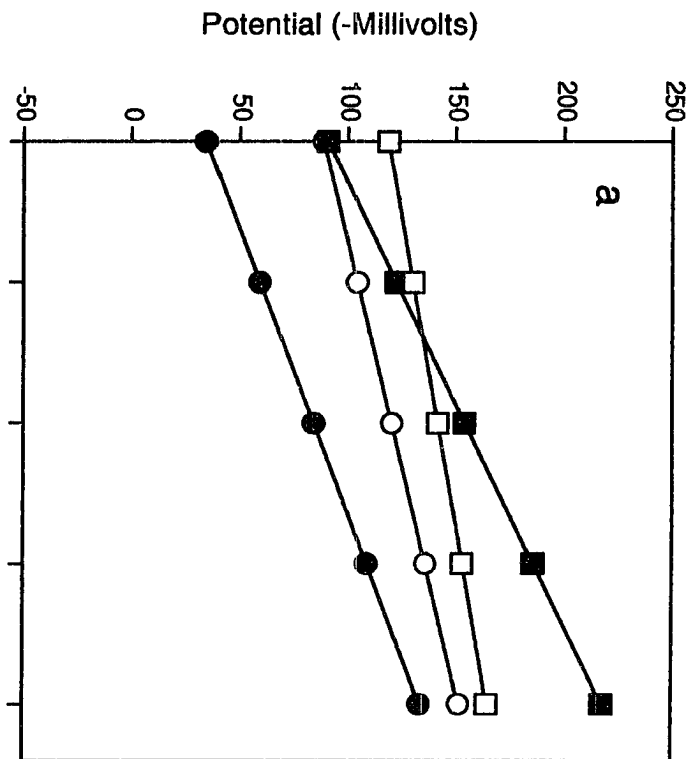


Figure 6: Response of  $\Delta p$  (a) and  $\Delta \Psi$  (b) during the growth phase of batch fermentations with and without pH control. Lines were generated from a linear regression done separately for each pH and fermentation condition. The % highest absorbance was determined from absorbance at the sample time compared to the highest absorbance attained by that culture. The  $\Delta p$  values (a) differed between the cultures with and without pH control ( $P < 0.01$ ). The  $\Delta \Psi$  values (b) differed between cultures grown at different pH values ( $P < 0.01$ ). Symbols:  $\circ$ , pH 7 with pH control;  $\square$ , pH 7 without pH control;  $\bullet$ , pH 5 with pH control; and  $\blacksquare$ , pH 5 without pH control.



## **RESPONSE OF *PROPIONIBACTERIUM THOENII* P127 TO ACID EXPOSURE: ADAPTATION AND MANIPULATION OF THE COMPONENTS OF THE PROTON MOTIVE FORCE**

A paper prepared for submission to Applied and Environmental Microbiology

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### **Abstract**

Logarithmically growing cells of *Propionibacterium thoenii* P127 that were exposed to mild acid conditions (pH 5.5) for one doubling were respectively 100 and 1000 times more resistant after 90 and 120 min of exposure to lethal acid conditions (pH 3.5) than were cells shifted directly from neutral pH to pH 3.5. This acid tolerance response required protein synthesis. The adapted (pH 5.5) cells were able to maintain a  $\Delta p$  (proton motive force) after 120 min of exposure to pH 3.5, while the  $\Delta p$  of unadapted (pH 7.0) cells had collapsed after 120 min at pH 3.5. When logarithmically growing cells were exposed to increased lactate concentrations at acidic pH, the  $\Delta p$  (pH gradient) component of the  $\Delta p$  increased from -12 mV at pH 6.8 to -62 mV at pH 5.5. There was a concomitant decrease in  $\Delta \Psi$  (electrical potential) from -99 mV at pH 6.8 to 0 mV at pH 5.5. The  $\Delta \Psi$  then increased from -20 mV at pH 5.0 to -108 mV at pH 4.0, while the  $\Delta p$  decreased from -48 mV to -5 mV. The cells were able to maintain a near neutral

internal pH when external pH values decreased from 7.0 to 6.1; the internal pH then decreased with further decrease in external pH. Cells exposed to high lactic acid concentrations neutralized at pH 7.0 had  $\Delta p$  values between -114 mV and -120 mV; the  $\Delta p$  was comprised almost entirely of  $\Delta \psi$ . These results suggest that proton concentration rather than the lactate anion had the greatest influence on  $\Delta p$ . The cells may manipulate the components of the  $\Delta p$  to maintain a large transmembrane potential. In addition, P127 cannot grow at pH values below 5.0. This may suggest that the cells' inability to maintain a large enough  $\Delta p$  is responsible for growth inhibition.

## Introduction

Propionibacteria are fermentative organisms that produce propionic and acetic acid from the metabolism of lactate and/or carbohydrates. The primary industrial use of these organisms is as a starter culture for Swiss-type cheeses (9), but they are also used as a silage inoculum (4), probiotic agent (14), and in the production of vitamin B<sub>12</sub> and propionic acid (19). The propionibacteria are relatively sensitive to low pH and generally will not grow below pH 5.0 (20). A strain that is tolerant to low pH could be of economic importance as an improved silage inoculum or probiotic feed additive. In addition, growth at low pH may facilitate downstream processes to remove product acids from fermentation broth.

It has been found that previous exposure to mildly acidic conditions enables some organisms to increase their resistance to extreme acid conditions.

Foster and Hall (5, 6) discovered that when logarithmically growing *Salmonella typhimurium* cells at neutral pH were shifted to pH 5.8 for one doubling, the cells were 100 to 1000 times more resistant to subsequent strong acid challenge (pH 3.3) than were cells that were shifted directly from neutral pH to pH 3.3. This response was called the acid tolerance response (ATR). The adapted cells survived lethal acid conditions because of an enhanced ability to maintain an internal pH above 5.0. Unadapted cells had internal pH values below 5.0, and rapidly lost viability. The authors concluded that the ATR system enhanced pH-homeostatic mechanisms and that acid-induced death was the direct result of lowered internal pH.

The objectives of this work were to determine if cells of *Propionibacterium thoenii* P127 became more resistant to lethal acidic pH when first exposed to mildly acidic conditions; to determine the effect of acid adaptation on the components of the proton motive force; and to determine the effect of exposure to lactic acid at acidic and neutral pH on the proton motive force of logarithmically growing P127 cells.

## **Materials and Methods**

### **Organism and culture conditions**

*Propionibacterium thoenii* P127 was obtained from the culture collection of the Department of Food Science and Human Nutrition at Iowa State University. Growth medium was sodium lactate broth (NLB) which contained 1% trypticase

soy broth (BBL Microbiology Systems, Cockeysville, MD), 1% yeast extract (Difco, Detroit, MI), 1% sodium lactate 60% syrup (Fisher Scientific Co., Pittsburgh, PA), and 0.6% Tween 80 (Fisher). For experiments conducted at lowered pH values, NLB was acidified to the desired pH with lactic acid. Inocula used in all experiments had been previously transferred at least three times (1% inoculum) in NLB with incubation at 32°C for 24 h between transfers.

### **Acid challenge experiments**

The measurement of the acid tolerance response was based on the methods of Foster and Hall (5). Strain P127 was inoculated (1%) into 300 ml of NLB (pH 7) in a 500-ml screw-capped Erlenmeyer flask and incubated at 32°C until mid-log phase (absorbance of 0.3-0.4 at 600 nm). The culture was divided into 100-ml aliquots, each of which was centrifuged at 9000 x *g* at 4°C for 15 min and resuspended in 100 ml of NLB at pH 7, 5.9, or 5.5. These cultures were incubated at 32°C for one doubling (absorbance of 0.6-0.8 at 600 nm); approximate doubling times were 5, 7, and 9 h, respectively. The cultures were then centrifuged (9000 x *g* at 4°C for 15 min) and resuspended in NLB adjusted to pH 3.5. This pH had been previously determined to be lethal to P127 (19). Samples were taken at 15- to 30-minute intervals, diluted, and plated onto nonselective medium (NLA: NLB + 1.5% agar). The plates were incubated at 32°C in GasPak jars (BBL) with the GasPak anaerobic generating system for 5-7 days. The  $\Delta\text{pH}$  and  $\Delta\psi$  of the cultures were measured at sample times 0, 60, and 120 min.

### Acid exposure experiments

Continuous fermentation was conducted in a BioFlo 3000 bioreactor (New Brunswick Scientific, Edison, NJ) with a working volume of 840 ml. The dilution rate was  $0.14 \text{ h}^{-1}$  with yeast extract (0.1%) as the limiting nutrient. The temperature was controlled at  $32^{\circ}\text{C}$ , the agitation was 125 rpm, and the pH was controlled at pH 7 with 3 N NaOH. Samples were taken at various time points with at least one retention time between sampling times. The cells were centrifuged ( $9000 \times g$  at  $4^{\circ}\text{C}$  for 15 min) and resuspended either in fresh growth medium acidified with lactic acid, or in neutralized (pH 7) medium with increased lactate concentrations (55 to 120 mM). Resuspended cells were incubated for 30 min at  $25^{\circ}\text{C}$  before samples were taken for  $\Delta\Psi$  and  $\Delta\text{pH}$  measurements. The cells were exposed to each lactic acid concentration and pH at least twice with 2 replicates at each exposure time.

### Proton motive force determination

Estimation of the proton gradient ( $\Delta\text{pH}$ ) and the transmembrane electrical potential ( $\Delta\Psi$ ) was based on distribution of [ $^{14}\text{C}$ ]salicylic acid ( $2.5 \mu\text{M}$ ,  $0.125 \mu\text{Ci}$ ; Dupont NEN, Boston, MA) and [ $^3\text{H}$ ]tetraphenylphosphonium bromide ( $\text{TPP}^+$ ,  $2.25 \mu\text{M}$ ,  $0.5 \mu\text{Ci}$ ; Amersham Corp., Arlington Heights, IL), respectively (11, 17, 18, 21). The accumulation of salicylate and  $\text{TPP}^+$  was determined by the silicon oil centrifugation technique (20). Corrections for nonspecific binding of the probes were based on the extent of probe accumulation in cells treated with  $20 \mu\text{M}$  valinomycin (Sigma Chemical, Co., St. Louis, MO) and  $5 \mu\text{M}$  nigericin (Sigma) for 60 min. For internal volume measurements, cells were incubated with  $^3\text{H}_2\text{O}$



(1.25  $\mu\text{Ci}$ ; Amersham) and [ $^{14}\text{C}$ ]polyethylene glycol (PEG, 3.9  $\mu\text{M}$ , 0.25  $\mu\text{Ci}$ ; Amersham).

### Calculations

The internal volumes were calculated as outlined by Kashket (11) from the difference between the total ( $^3\text{H}_2\text{O}$  permeable) and extracellular ( $^{14}\text{C}$ -PEG nonpermeable) volume of pellets. The internal volume for the cells grown at pH 7 was calculated as  $0.96 \pm 0.13 \mu\text{l mg}^{-1}$  dry weight; the internal volume of the pH 5.5 culture was calculated as  $0.65 \pm 0.039 \mu\text{l mg}^{-1}$  dry weight.

The  $\Delta\text{pH}$  was calculated from the distribution ratio of [ $^{14}\text{C}$ ]salicylic acid using the Henderson-Hasselbalch equation (11, 17, 18, 21):

$$\Delta\text{pH} = \log \frac{[\text{salicylic acid}]_{\text{in}}}{[\text{salicylic acid}]_{\text{out}}}$$

When the pH of the medium was less than the  $\text{pK}_a$  (2.97) + 1.0 (i.e. less than 3.97) a correction factor was necessary (16, 18). In these cases, the  $\Delta\text{pH}$  was calculated by the following equation:

$$\Delta\text{pH} = \log \left\{ \left( \frac{[\text{salicylic acid}]_{\text{in}}}{[\text{salicylic acid}]_{\text{out}}} \right) \left( 10^{\text{pK}_a - \text{pH out}} + 1 \right) - 10^{\text{pK}_a - \text{pH out}} \right\}$$

The  $\Delta\Psi$  was calculated in a similar manner (11, 17, 18, 21):

$$\Delta\Psi = -Z \log \frac{[\text{TPP}^+]_{\text{in}}}{[\text{TPP}^+]_{\text{out}}}$$

Where  $Z = 59 \text{ mV}$  at  $25^\circ\text{C}$ . The proton motive force was calculated as (11, 17, 18, 21):

$$\Delta p = \Delta\Psi - Z\Delta\text{pH}$$

By convention, negative signs are used for  $\Delta\Psi$  (negative inside) and  $\Delta p$  (negative and alkaline inside as compared to the external medium). In discussions comparing the  $\Delta\Psi$  and  $\Delta pH$ ,  $Z\Delta pH$  is used because multiplication by  $Z$  converts the  $\Delta pH$  to the same units (millivolts) as  $\Delta\Psi$ .

## Results and Discussion

### Acid adaptation

To determine if *P. thoenii* P127 exhibited an acid tolerance response, the viability of P127 in NLB at pH 3.5 was compared after prior growth at pH 7, 5.9, and 5.5. We choose pH 5.9 and 5.5 as the adaptive pH values because they were mild acid stress conditions for P127. At values below pH 5.5, growth was more severely inhibited (20). In addition, previous researchers (5, 6, 7) had found that pH 5.8 to 6.0 induced the acid tolerance response in *S. typhimurium*.

The results (Figure 1) show that although all cultures experienced a loss of viability, the adapted cultures were better protected from acid death relative to the pH 7 culture. The doubling at pH 5.9 provided some protection, but not as much as did the growth at pH 5.5. This suggests that different levels of adaptation may occur as the cells are exposed to greater acid stress. Foster and Hall (5) found that the induction of ATR in *S. typhimurium* was not an all-or-nothing phenomenon; adaptation at pH values from 6.3 to 5.7 resulted in a graded ATR.

Because of the higher survival rate (100 times) of cultures exposed to pH 5.5 vs. pH 5.9, pH 5.5 was chosen as the adaptation pH for subsequent

experiments. Prior exposure to pH 5.5 allowed 1000 times higher survival rate after 120 min of exposure to pH 3.5 compared to the unadapted (pH 7) culture.

To determine if adaptation would protect the cells from an even greater acid challenge, both adapted and unadapted cultures were resuspended in NLB adjusted to pH 3.0 and pH 2.5 (Table 1). Both cultures experienced a one-log reduction in viable counts immediately after exposure to pH 3.0; after 20 min the viable counts for both cultures were below the detection limit. In addition, viable cells were below the detection limit for both cultures immediately after exposure to pH 2.5.

Foster and Hall (5) offered two possible explanations for the development of acid tolerance: growth at mild acid stress could have resulted in a physiological alteration in the cell which made it less susceptible to acid damage, or acid tolerance could be a molecular process that required genetic regulation and synthesis of new proteins. Protein synthesis was determined to be involved in the induction of the ATR system in *S. typhimurium* (5, 13).

To determine if protein synthesis was necessary for acid adaptation of P127, chloramphenicol ( $100\ \mu\text{g ml}^{-1}$ ) was added to the original culture 2.5 h prior to the point of adaptation; chloramphenicol was also present during the adaptation period. Chloramphenicol was previously determined to be nonlethal to P127 at  $100\ \mu\text{g ml}^{-1}$  (data not shown). The adapted (pH 5.5) culture showed 27% survival after 60 min of exposure to pH 3.5 (Table 2). In contrast, in the presence of chloramphenicol the culture was much more susceptible to acid damage, with <0.01% survival. Unadapted cultures with and without chloramphenicol experienced a significant loss of viability after 60 min of pH 3.5 exposure (Table

2). The results indicate that protein synthesis is required for acid adaptation to occur in P127.

### **Proton motive force during acid challenge**

Acid damage caused rapid loss of viability in *S. typhimurium* cells when the internal pH dropped below 5.0 (6). The researchers concluded that adapted cells survived the acid challenge (pH 3.3) better than did unadapted cells because of their enhanced ability to maintain an internal pH above 5.0. To determine if acid adaptation affected the internal pH homeostatic mechanisms of P127, the  $\Delta\text{pH}$  and  $\Delta\Psi$  were measured after 0, 60, and 120 min of acid challenge (pH 3.5) for both adapted and unadapted cultures (Table 3). The unadapted cells were able to maintain a  $\Delta\text{p}$  at time 0 (-49 mV) and 60 min (-61 mV) but not at 120 min. In contrast, the adapted cells were able to maintain a large  $\Delta\text{p}$  throughout the acid challenge: -76, -85, and -72 mV at 0, 60, and 120 min, respectively.

The internal pH (external pH +  $\Delta\text{pH}$ ) value of the cells of the unadapted culture were higher at time 0 (pH 3.93) than was that of the adapted cells (pH 3.58) (Table 4). After 120 min at pH 3.5, the  $\Delta\text{pH}$  of the unadapted cells had collapsed. In contrast, the  $\Delta\text{pH}$  of adapted cells increased after 60 min of acid challenge. The transient low  $\Delta\text{pH}$  immediately after exposure to pH 3.5 may result from slow response of the primary proton pumps, by whose action the cell regulates its internal pH. Previous researchers have found that upon shifting *E. coli* cells from pH 7.2 to more alkaline conditions, a transient decrease in  $\Delta\text{pH}$  and an increase in  $\Delta\Psi$  were seen before steady state values were established by the pH homeostatic mechanisms (18).

The adapted P127 cells may have survived at pH 3.5 better than the unadapted cells because of their ability to maintain a  $\Delta\text{pH}$ . The internal pH values of *P. thoenii* were lower than those measured previously in *S. typhimurium*; this may be because fermenting bacteria such as the propionibacteria can tolerate higher internal proton concentrations than can other bacteria (12).

### Acid exposure

To determine the effect of acid on the proton motive force of P127, logarithmically growing cells were harvested from continuous culture and exposed to NLB acidified to pH values ranging from 7.0 to 4.0 with lactic acid. Continuous culture was used so that the cells were in the same physiological state at different sampling times; this should reduce error due to differences in growth stage.

The  $\Delta\text{pH}$  increased from -12 mV at pH 6.8 to -62 mV at pH 5.5 (Figure 2a). In the same cultures, the  $\Delta\Psi$  values decreased from -99 mV to 0. This is consistent with previous observations in bacteria that the contribution of  $\Delta\Psi$  and  $\Delta\text{pH}$  to  $\Delta p$  are dependent on the pH of the medium (2, 12). The  $\Delta\Psi$  was the major component at neutral pH values, while the  $\Delta\text{pH}$  was the major component at acidic pH values. The  $\Delta\Psi$  then increased from -20 mV at pH 5.0 to -108 mV at pH 4.0, while the  $\Delta\text{pH}$  decreased from -48 mV to -5 mV. The large decrease in  $\Delta\text{pH}$  below pH 5.0 suggests that the cell membranes might have lost their impermeability to protons and thus were not able to maintain a pH gradient in the presence of high concentrations of protons. The concomitant increase in  $\Delta\Psi$  with the decrease in  $\Delta\text{pH}$  is consistent with observations in other bacteria (2, 12).

Researchers have determined that changes in the proton gradient were compensated for by corresponding changes in the electrical gradient; by this mechanism a constant  $\Delta p$  could be maintained (2, 12, 18)

To investigate further the cells' ability to increase one component of the  $\Delta p$  when the other decreases, the  $\Delta pH$  was measured while the  $\Delta \Psi$  was reduced with valinomycin (20  $\mu M$ ). Valinomycin is an ionophore that catalyzes the electrical uniport of cations (especially  $K^+$ ) and depletes the  $\Delta \Psi$  (17). Conversely, the  $\Delta \Psi$  was measured while the  $\Delta pH$  was reduced with nigericin (5  $\mu M$ ), an ionophore that carries out the antiport of  $K^+$  for  $H^+$  and dissipates the  $\Delta pH$  in an electroneutral manner (17). When the  $\Delta pH$  was depleted, the  $\Delta \Psi$  increased for cultures at acidic and neutral pH; the most dramatic increase occurred at pH 5 (Table 5). In contrast, the  $\Delta pH$  dramatically increased at neutral pH when the  $\Delta \Psi$  had been dissipated.

These results suggest that the cells manipulate the components of the  $\Delta p$  to maintain a large transmembrane potential. When the major component of the  $\Delta p$  was reduced, i.e.  $\Delta pH$  at pH 5 and  $\Delta \Psi$  at pH 7, the cells dramatically increased the remaining component. However, when the secondary component of the  $\Delta p$  was reduced, i.e.  $\Delta \Psi$  at pH 5 and  $\Delta pH$  at pH 7, there was only a small increase in the remaining component. This confirms the importance of the  $\Delta pH$  at low pH and  $\Delta \Psi$  at neutral pH in the maintenance of  $\Delta p$ .

### **Internal pH values**

Cells exposed to media with decreasing pH values were able to maintain a near neutral internal pH when the external pH ranged from 6.8 to 6.1 (Table 6).

The internal pH then decreased with further decrease in external pH. Previous observations with other fermentative organisms including *Clostridium thermoaceticum* (1), *Sarcina ventriculi* (8), and lactic acid bacteria (3, 12, 15, 16, 23) demonstrated that the internal pH of these organisms decreased as the external pH declined.

The most dramatic change in internal pH, from 5.9 to 4.7, occurred when the pH of the medium decreased from 5.1 to 4.5. Previously we found that strain P127 could not initiate growth in medium acidified below pH 5.0 with lactic acid (20) and considered pH 5.0 to be the critical external pH threshold for this strain. The large decline in internal pH that occurred at external pH values below 5.0 suggests that the cells' inability to maintain a large enough  $\Delta\text{pH}$  at these conditions is responsible for growth inhibition. Furthermore, we had observed that the critical internal pH threshold for P127 was 5.8 (20). The internal pH values measured in this study are in agreement with this value.

We previously observed that strain P127 remained viable but could not grow at pH 4.5; viability was lost at pH 4.1 (20). In the current study we measured an internal pH of 4.7 when the external pH was 4.5. This internal pH did not allow the cells to grow, but was not lethal. At external pH 4.0, the  $\Delta\text{pH}$  of the cells was very small, and the internal pH was 4.1. These results suggest that the cells need to maintain an internal pH above 4.1 to remain viable.

### **Effect of lactate**

The  $\Delta\text{p}$  values for cells exposed to increased lactate at neutral pH ranged from -111 mV to -126 mV and were primarily composed of  $\Delta\Psi$  (Figure 2). The

cells maintained a neutral internal pH at all lactate concentrations (Table 6). In addition, the  $\Delta\psi$  and  $\Delta\text{pH}$  values measured in the presence of nigericin and valinomycin were not significantly affected by lactate concentrations (Table 5). These results suggest that the cells were primarily affected by the proton concentration and not by the lactate anion.

This study has shown that strain P127 undergoes some adaptation when grown at pH 5.5. We propose that protein synthesis and the ability to maintain a  $\Delta\text{pH}$  are necessary for this adaptation. Researchers have shown that the synthesis of outer membrane proteins in *S. typhimurium* was involved in acid adaptation (13). These proteins may have increased the cell membrane's impermeability to protons, which would allow the cells to survive better at lethal acidic pH conditions. Investigation of the proteins that were synthesized by P127 during growth at pH 5.5 may help to elucidate the adaptation process.

Growth inhibition of strain P127 below pH 5 appears to be due to its inability to maintain its internal pH at or above pH 5.8. Similar values have been found for lactic acid bacteria (15, 16, 22). Nannen and Hutkins (16) found that growth and metabolic inhibition of *S. thermophilus*, *L. lactis* ssp. *lactis*, and *L. lactococcus* ssp. *cremoris* occurred below an external pH of 5.0 as a result of low internal pH (between pH 5.0 and 5.5). However, the acid-tolerant organism *L. casei* was able to maintain an internal pH between 5.1 and 6.4, even at external pH values as low as 3.8. Thus, for P127 to increase its acid tolerance, it would need to increase its ability to maintain a large pH gradient. This would involve several changes in the cell: increases in membrane impermeability to protons, increases in activity of the proton pumps, or decreases in the pH optima of internal



enzymes and proton pumps. Thus, isolation and/or development of a variant of P127 that could grow below pH 5 involves several mechanisms and would likely be difficult.

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Table 1: Viable counts (cfu ml<sup>-1</sup>) of unadapted (pH 7) and adapted (pH 5.5) P127 cultures after exposure to pH 3.0 and pH 2.5.

Culture	Original <sup>a</sup>	pH 3.0			Original <sup>a</sup>	pH 2.5	
		0 min	10 min	20 min		0 min	10 min
pH 7.0	4.7x10 <sup>7</sup>	3.2x10 <sup>6</sup>	<10 <sup>1</sup>	<10 <sup>1</sup>	4.7x10 <sup>7</sup>	<10 <sup>1</sup>	<10 <sup>1</sup>
pH 5.5	2.3x10 <sup>8</sup>	4.5x10 <sup>7</sup>	1.5x10 <sup>3</sup>	<10 <sup>1</sup>	1.4x10 <sup>8</sup>	<10 <sup>1</sup>	<10 <sup>1</sup>

<sup>a</sup> Viable cell counts in cfu ml<sup>-1</sup> of culture prior to acid challenge

Table 2: Effect of growth with chloramphenicol (100 µg ml<sup>-1</sup>) on acid adaptation of P127. Percent survival was determined 60 min after resuspending unadapted (pH 7.0) and adapted (pH 5.5) cultures in NLB acidified to pH 3.5 with lactic acid. Values are averages of 3 experiments.

Culture	Chloramphenicol	% Survival
Unadapted	-	0.04
Unadapted	+	<0.01
Adapted	-	26.6
Adapted	+	<0.01

Table 3: Components of the proton motive force for unadapted (U) and adapted (A) cells during acid challenge at pH 3.5.

Time (min)	$\Delta p$ (mV)		$\Delta \Psi$ (mV)		$Z\Delta pH$ (mV)	
	U <sup>a</sup>	A <sup>b</sup>	U <sup>a</sup>	A <sup>b</sup>	U <sup>a</sup>	A <sup>b</sup>
0	-49	-76	-24	-65	-25	-11
60	-61	-85	-39	-50	-22	-35
120	0	-72	0	-31	0	-41

<sup>a</sup> unadapted (pH 7.0) cells

<sup>b</sup> adapted (pH 5.5) cells

Table 4: Internal and external pH values for unadapted and adapted cells during acid challenge at pH 3.5.

Time (min)	Unadapted			Adapted		
	pH out <sup>a</sup>	$\Delta pH$	pH in <sup>b</sup>	pH out <sup>a</sup>	$\Delta pH$	pH in <sup>b</sup>
0	3.50	0.43	3.93	3.40	0.18	3.58
60	3.50	0.37	3.87	3.50	0.60	4.10
120	3.60	0	3.60	3.50	0.70	4.20

<sup>a</sup> pH out = external pH

<sup>b</sup> pH in = internal pH (external pH +  $\Delta pH$ )

Table 5: Comparison of the  $\Delta\Psi$  and  $Z\Delta pH$  values at different pH and lactate concentrations for cells of P127 treated with nigericin (5  $\mu M$ ) or valinomycin (20  $\mu M$ ) to dissipate the  $\Delta pH$  or  $\Delta\Psi$ .

pH	Lactate (mM)	Nigericin	Valinomycin	$\Delta\Psi$ (mV)	$Z\Delta pH$ (mV)
6.83	55	+	-	-134	-
6.96	70	+	-	-132	-
5.07	70	+	-	-128	-
6.83	55	-	+	-	-55
6.96	70	-	+	-	-48
5.07	70	-	+	-	-53
6.78	55	-	-	-99	-12
6.96	70	-	-	-115	-11
5.06	70	-	-	-20	-48

Table 6: External and internal pH values for cells exposed to increased lactate at acidic and neutral pH values.

Lactate (mM)	Acidic pH			Neutral pH		
	pH out <sup>a</sup>	$\Delta pH$	pH in <sup>b</sup>	pH out <sup>a</sup>	$\Delta pH$	pH in <sup>b</sup>
55	6.78	0.21	6.99	6.78	0.20	6.98
56	6.45	0.74	7.19	6.96	0.11	7.07
59	6.06	0.94	7.00	7.00	0.05	7.05
61	5.52	1.05	6.57	6.94	0.10	7.04
70	5.06	0.82	5.88	6.96	0.18	7.14
83	4.48	0.25	4.73	6.96	0.09	7.05
120	4.00	0.09	4.09	6.90	0.31	7.21

<sup>a</sup> pH out = external pH

<sup>b</sup> pH in = internal pH (external pH +  $\Delta pH$ )

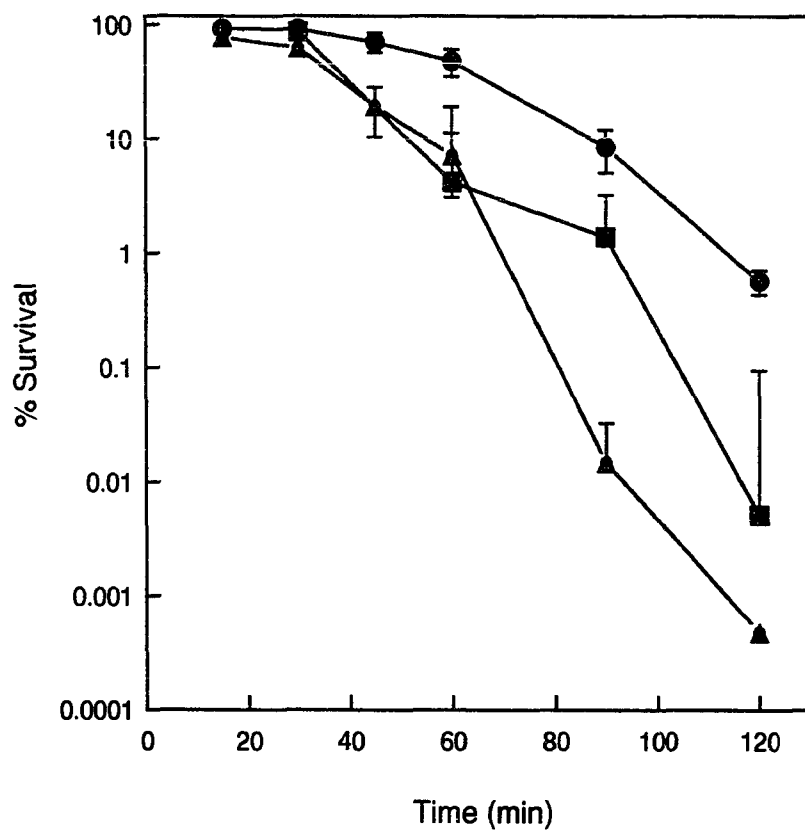


Figure 1: Survival of cultures of P127 with time of exposure to pH 3.5. Survivors were enumerated on nonselective medium. Values are the average of 5 experiments with standard error values shown. Symbols:  $\blacktriangle$  unadapted culture grown at pH 7,  $\blacksquare$  culture adapted at pH 5.9, and  $\bullet$  culture adapted at pH 5.5.

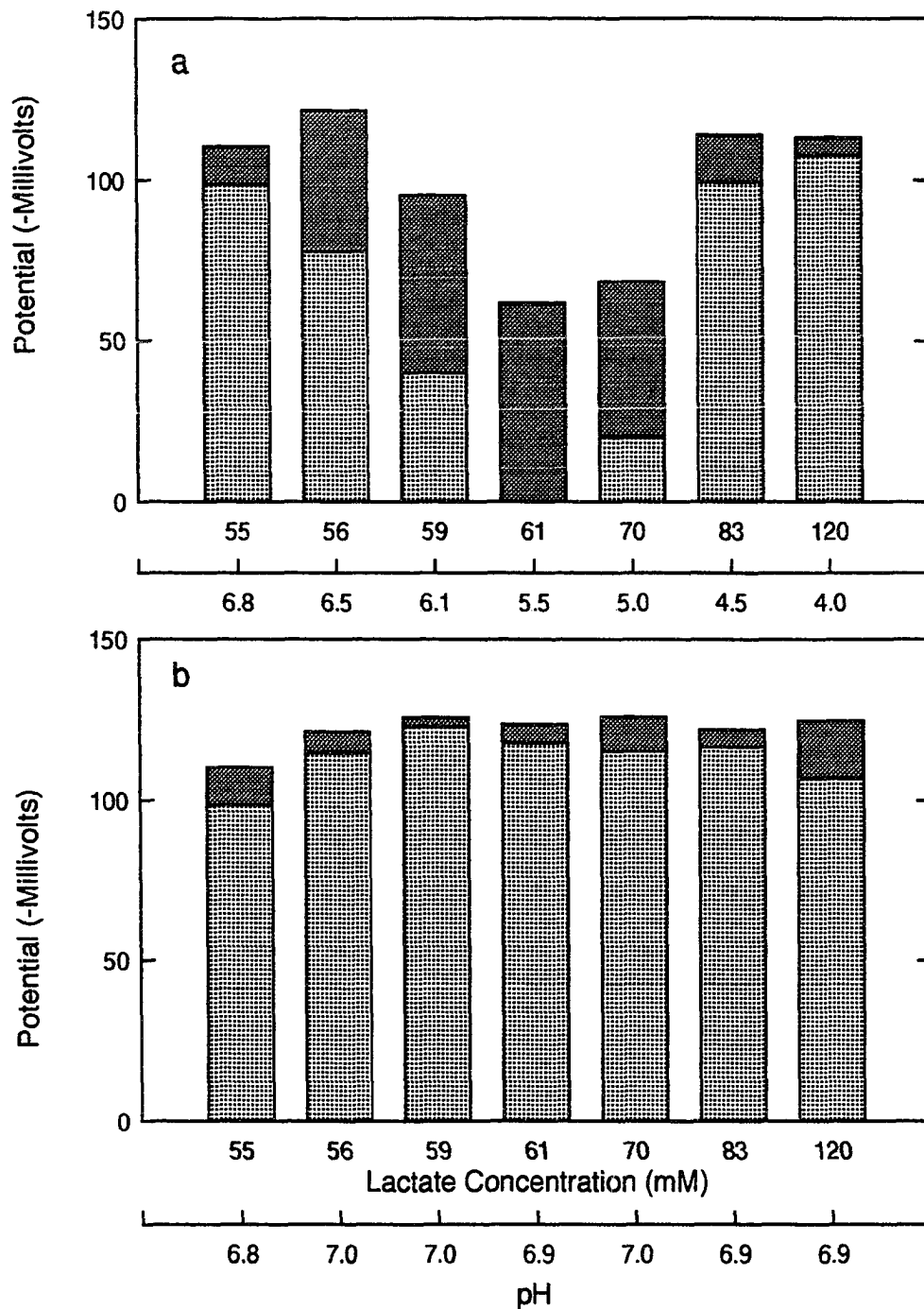


Figure 2: The  $\Delta\Psi$  and  $Z\Delta pH$  of P127 after exposure to increased lactate at acidic pH (a) and neutral pH (b). Values are the averages of duplicate exposures with 2 replicates at each exposure time.  
 Symbols:  $\Delta\Psi$   $Z\Delta pH$ .



## GENERAL CONCLUSIONS

It has been recognized that the optimum pH for growth of propionibacteria is between pH 6.0 and 7.1, and that growth rates decline at acidic pH (41). However, studies have not been conducted to explain these effects. Experiments reported in this research define the parameters of growth at low pH and help to explain the mechanism of acid- and low-pH-tolerance and inhibition.

Six strains of *Propionibacterium* (three strains of *P. acidipropionici*, one strain of *P. jensenii*, and two strains of *P. thoenii*) were identified as potential acid- and low-pH-tolerant strains. These strains were selected based on their ability to produce large amounts of propionic and acetic acids and obtain a final pH <4.4 at the end of culture growth with glucose, fructose, and maltose as carbon sources. The *P. acidipropionici* strains produced lower final pH values and on average higher acid concentrations than did the other strains.

The lowest pH values at which the strains could initiate growth and the lowest pH values at which they could remain viable were determined with lactic, propionic, and hydrochloric acids as acidulants. Generally, the strains could not grow below pH 5 in the presence of organic acids; however, they could remain viable at significantly lower pH values (down to pH 4.1) for 48 h. All strains were more pH-tolerant when the inorganic acid was used. The *P. thoenii* and *P. jensenii* strains were able to initiate growth and survive at lower pH values than could the *P. acidipropionici* strains. However, the *P. thoenii* and *P. jensenii* strains were more sensitive to increased propionate concentrations at neutral pH

than were the *P. acidipropionici* strains. Thus, the ability to produce and tolerate large amounts of acids was not directly related to a strain's ability to initiate growth or survive in low-pH conditions. It appeared that the response to acid anions and to protons was governed by two different mechanisms.

To determine how acid production and low pH affected *P. thoenii* P127, the proton motive force ( $\Delta p$ ) of P127 was measured during batch growth at pH 7 and pH 5. The results demonstrated that the contribution of the individual components, pH gradient ( $\Delta pH$ ) and electrical potential ( $\Delta \Psi$ ), to the overall  $\Delta p$  depended on the pH of the medium. At pH 7, the  $\Delta p$  was comprised of only the  $\Delta \Psi$ ; the internal pH remained constant at pH 7. During growth at pH 5, the  $\Delta p$  consisted of both  $\Delta \Psi$  and  $\Delta pH$  and the internal pH decreased slowly throughout the fermentation with acid production.

The acid tolerance of fermentative organisms had been attributed to their ability to lower their internal pH as the external pH decreased in the presence of weak organic acids (15, 79, 80, 81, 82). Weak organic acids diffuse across the membrane in accordance with the pH gradient and dissociate at the more alkaline internal pH. The impermeable anion accumulates internally, at concentrations predicted by the Henderson-Hasselbalch equation; this accumulation is believed to contribute to inhibitory effects of the acid. Strain P127 was able to prevent large amounts of propionic and acetic acid from accumulating internally by lowering its  $\Delta pH$ . However, a lower  $\Delta pH$  resulted in a lower internal pH; this can also contribute to organic acid inhibition (7, 46, 69, 82).

When the internal pH of P127 fell below pH 5.8, the rate of consumption of substrate lactate declined and the cells entered death phase, even though the

lactate had not yet been depleted. These results suggest that pH 5.8 may be the critical internal pH threshold for strain P127. Cellular functions are inhibited at internal pH values below the critical threshold. Most internal enzymes have a narrow range in which they are active (7, 46, 69); pH 5.8 may be the lower limit for enzymes in this strain.

The ATP concentrations of P127 grown in batch culture controlled at pH 7 and pH 5 were measured. Throughout the entire fermentation, the ATP concentrations for the pH 5 culture were significantly lower than for the pH 7 culture. This may be due to the reverse action of the  $H^+$ -ATPase which consumes ATP to expel protons in order to maintain an alkaline internal pH with respect to the medium (4, 7, 67). At pH 5, strain P127 decreased its intracellular pH, lactate consumption rate, and intracellular ATP concentration. These results suggest that energy availability may contribute to the pH sensitivity of P127.

When strain P127 was exposed to increased lactate concentrations at acidic pH, the cells manipulated the components of the  $\Delta p$  to maintain a large transmembrane potential. The cells maintained a near neutral internal pH when the external pH ranged from 6.8 to 6.1. However, the internal pH then decreased with further decrease in the external pH. A large decline in internal pH occurred at external pH values below 5.0; this may suggest that growth inhibition was due to the inability of the cell membranes to remain impermeable to protons.

Strain P127 remained viable but could not grow at pH 4.5; viability was lost at pH 4.1. At an external pH of 4.5, the corresponding internal pH was measured as 4.7. This internal pH did not allow the cells to grow, but was not lethal. At pH

4.0, the  $\Delta\text{pH}$  for cells was very small, and the internal pH was 4.1. These results suggest that the cells need to maintain an internal pH above 4.1 to remain viable.

Examination of transmission electron micrographs of P127 cells grown at pH 7 and pH 5 showed differences in cell wall and membrane structure at the two conditions. At pH 5, cell walls appeared diffuse and seemed to lack a definitive structure. Since the cells were fixed in buffers adjusted to the pH at which they were grown, it is possible that the difference in appearance may be due to pH-induced differences in the ability of the cells to take up stain. Examination of cells grown at pH 5 and fixed at pH 7 would test this possibility and help to answer the question of whether cell structure was altered by growth at low pH. In addition, a comparison of the capsules of cells grown at pH 7 and pH 5 may reveal other morphological changes at low pH.

The initial goal of this study was to isolate and/or develop an acid- and low-pH-tolerant variant that could be used as a silage inoculant, as a probiotic feed additive, or as a more productive strain for industrial fermentations. The development of a strain tolerant to both acid and low pH seems unlikely, however; tolerance to acid and to acid anions appeared to be two separate complex mechanisms. Indeed, the propionate-tolerant variant of P9, strain P200910, had been isolated after repeated exposure to increasing propionate concentrations at neutral pH (91), but showed the least tolerance to low pH.

The development of a fermentative low-pH-tolerant variant would require changes in several characteristics of the strain. A strain could increase its pH-tolerance by maintaining a more neutral internal pH. In this way, the internal enzymes could remain active. This would involve the overexpression of proton

pumps such as the  $H^+$ -ATPase. However, this would require that the cell have a large  $\Delta pH$ ; this would allow more of the product acids of the fermentation to accumulate inside the cell.

A strain could also increase its pH-tolerance by lowering the pH optima of its internal enzymes. Such a strain could maintain a lower  $\Delta pH$  and would not accumulate as high an internal concentration of acid anions. However, the pH optimum of an enzyme depends on several factors, and changing the pH range of activity would not be easily accomplished. One approach could be through genetic manipulation of the enzyme's primary structure, to contain more basic amino acids. This would involve locating the active site(s), determining the amino acid residues involved in catalysis, and using site-directed mutagenesis to alter these residues. A strain could also be altered by random mutagenesis using a chemical mutagen followed by selection at low pH.

In addition, either approach to obtaining a low-pH-tolerant variant would require changes in the cell membranes to make them more impermeable to protons. Considering the complex mechanisms involved, it seems unlikely that a strain of *Propionibacterium* is a good candidate for low-pH-tolerance.

An alternative approach would be to gradually adapt the cells to low pH by exposing them to mildly acidic conditions. When logarithmically growing cells of P127 were exposed to a mild acid stress (pH 5.5) for one doubling, they were more resistant to a subsequent lethal pH exposure (pH 3.5) than were cells that were shifted directly from neutral pH to pH 3.5. We proposed that protein synthesis and the ability to maintain a  $\Delta pH$  were necessary for adaptation. The

unadapted cells had an internal pH of 3.9 upon exposure to pH 3.5, while the adapted cells maintained an internal pH of 4.2.

Researchers have shown that the synthesis of outer membrane proteins and the activity of the H<sup>+</sup>-ATPase in *S. typhimurium* were involved in acid adaptation (27, 28, 53). The membrane proteins may have increased the cell membrane's impermeability to protons, which allowed the cells to survive better at lethal acidic pH conditions. Investigation of the proteins that were synthesized upon acid exposure may further explain the adaptation process in P127. In addition, isolation of H<sup>+</sup>-ATPase mutants of P127 would help determine the role of this enzyme in adaptation.

Other areas for future research include:

1) Measurement of the  $\Delta p$  of strain P127 during fed-batch fermentation.

This would determine better how acid production affects this strain because in the presence of excess carbon source the culture would produce more acid than did the batch culture. This experiment would allow us to determine the propionic acid concentration at which the cell ceases growth and fermentation.

2) Isolation and characterization of the H<sup>+</sup>-ATPase from unadapted and adapted cells of P127. The pH optima and basal activities of these enzymes would help explain the low-pH adaptation response.

3) Comparison of the components of the  $\Delta p$  of strain P9 and its propionate-tolerant variant, P200910, when these strains were grown and exposed to increased propionate concentrations. This would help explain the mechanism of propionate anion tolerance.

4) Isolation and characterization of membranes of cells grown at neutral and low pH. The composition of fatty acids, proteins, and lipids can then be determined for cells grown at neutral and low pH.

In addition, the development of the protocol for the measurement of the  $\Delta p$  will allow our laboratory to determine sugar transport systems in propionibacteria and the mode of action of the bacteriocins produced by strains of propionibacteria. Determination of sugar transport systems is accomplished by incubating cells in the presence of radiolabeled sugars and measuring the internal concentration of the sugar over time. A comparison of the  $\Delta p$ , internal concentrations, and rates of transport of the sugar for cells incubated in the presence and absence of ATPase inhibitors, protonophores, ionophores, and metabolic inhibitors would determine if transport was active or passive. The mode of action of the bacteriocins can be determined by measuring the components of the  $\Delta p$  of an indicator organism in the presence and absence of the bacteriocin.

## APPENDIX A: PROTON MOTIVE FORCE CALCULATIONS

### Internal Volume Calculations

The internal volume of cells was determined from the difference between the total and extracellular volumes of the cell pellets. The total volume was measured with  $^3\text{H}_2\text{O}$  (1.25  $\mu\text{Ci}$ ), and the extracellular volume was measured with [ $^{14}\text{C}$ ] PEG (3.9  $\mu\text{M}$ , 0.25  $\mu\text{Ci}$ ). A stock solution of  $^3\text{H}_2\text{O}$  (0.25  $\mu\text{Ci}/\mu\text{l}$ ) was made from 0.25 ml of  $^3\text{H}_2\text{O}$  (5  $\mu\text{Ci}/\mu\text{l}$ ) and 0.475 ml of “cold”  $\text{H}_2\text{O}$ ; PEG was used as purchased. The internal volume was measured at different times throughout culture growth; each sample time consisted of triplicate measurements. The internal volume was calculated as follows.

Total volume of the cell pellet ( $V_t$ )  $^3\text{H}_2\text{O}$  counts:

$$V_t = \frac{\text{DPM } [^3\text{H}]_{\text{pellet}} - \text{DPM Bkgd}}{\text{DPM } [^3\text{H}]_{\text{supernatant}} - \text{DPM Bkgd}}$$

Where DPM Bkgd is the counts of a nonradioactive control culture.

Extracellular volume of the cell pellet ( $V_e$ ) [ $^{14}\text{C}$ ] PEG counts:

$$V_e = \frac{\text{DPM } [^{14}\text{C}]_{\text{pellet}} - \text{DPM Bkgd}}{\text{DPM } [^{14}\text{C}]_{\text{supernatant}} - \text{DPM Bkgd}}$$



Internal volume ( $V_{in}$ ) ( $\mu\text{l mg}^{-1}$  dry weight):

$$V_{in} = \frac{(\text{avg } V_t - \text{avg } V_e)(\text{volume of supernatant } (\mu\text{l}))}{\text{pellet weight (mg)}}$$

In the initial internal volume measurements [ $^{14}\text{C}$ ] inulin (0.5  $\mu\text{Ci}$ ) was used to measure the extracellular volume. However, there were large inconsistencies among the calculated internal volumes at each sampling time, including some negative values. After consulting with Dr. Thomas Montville (Rutgers, The State University of New Jersey) and Dr. Herbert Strobel (University of Kentucky), we tried PEG instead of inulin. Both consultants suggested that inulin was such a large molecule that sometimes it may adhere to cells. Also, bacteria behave differently and thus not all probes worked for every organism. Dr. Strobel's lab uses PEG or nontransported amino acids to avoid the binding of the large molecules. After we began using PEG, we no longer measured negative values for internal volumes.

The internal volume was measured at various stages of culture growth for cultures grown at pH 7 and pH 5 (Table A1). The average internal volume at pH 7 was  $0.96 \pm 0.13 \mu\text{l mg}^{-1}$  dry weight and at pH 5 it was  $0.51 \pm 0.068 \mu\text{l mg}^{-1}$  dry weight. At pH 5.5 for the acid tolerance response experiment, internal volume was  $0.65 \pm 0.039 \mu\text{l mg}^{-1}$  dry weight. Although the volume appeared to decrease with culture age, all values were used to calculate the average. Small changes in internal volume do not have a significant effect on the final  $\Delta\Psi$  and  $\Delta\text{pH}$  calculations, which are calculated as the logarithm of the concentration ratios. The  $\Delta\Psi$  and  $\Delta\text{pH}$  values for the pH 7 and pH 5 cultures differed by only 14

mV when they were calculated with the average internal volume +/- two standard deviations (1.22 and 0.70  $\mu\text{l mg}^{-1}$  dry weight for pH 7 and 0.65 and 0.27  $\mu\text{l mg}^{-1}$  dry weight for pH 5 cultures).

Table A1: Calculated internal volumes of P127 at various stages of growth at pH 7 and pH 5.

pH	Time (h)	Internal Volume ( $\mu\text{l mg}^{-1}$ dry weight)
7	24	1.16
7	24	1.14
7	72	0.95
7	96	0.82
7	96	0.91
7	96	0.90
7	C.C. <sup>a</sup>	0.86
7	C.C. <sup>a</sup>	0.97
5	48	0.47
5	72	0.59
5	96	0.52
5	168	0.41
5	168	0.57
5	666	0.47
5.5	ATR <sup>b</sup>	0.60
5.5	ATR <sup>b</sup>	0.68
5.5	ATR <sup>b</sup>	0.65
5.5	ATR <sup>b</sup>	0.67

<sup>a</sup> C.C. = continuous culture experiment

<sup>b</sup> ATR = acid tolerance response experiment, cells were grown at pH 7 until mid-logarithmic phase and then for one doubling at pH 5.5.

### Nonspecific Binding Calculations

The probes that are used to estimate the  $\Delta\psi$  (tetraphenylphosphonium, TPP<sup>+</sup>) and  $\Delta\text{pH}$  (salicylic acid) dissociate in solution and are charged. Charged compounds will bind to cell membranes and thus corrections need to be made to the concentration ratios of these probes. The correction factor was measured from the amount of probe that accumulated in cells that had been incubated for 60 min with nigericin (5  $\mu\text{M}$ ), valinomycin (20  $\mu\text{M}$ ), and 40 mM K<sup>+</sup> (added as K<sub>2</sub>HPO<sub>4</sub>). Nigericin carries out the symport of H<sup>+</sup> and K<sup>+</sup>; this dissipates the  $\Delta\text{pH}$ . Valinomycin catalyzes the electrical uniport of cations (especially K<sup>+</sup>) and thus dissipates the  $\Delta\psi$ . The nonspecific binding (NSB) correction factors were calculated from:

$$\text{Probe}_{\text{bound}} (\text{DPM} / \mu\text{l}) = \frac{\text{DPM pellet} - \text{DPM Bkgd}}{[\text{internal volume} (\mu\text{l}/\text{mg})][\text{pellet weight} (\text{mg})]}$$

$$\text{Probe}_{\text{free}} (\text{DPM}/\mu\text{l}) = \frac{\text{DPM supernatant} - \text{DPM Bkgd}}{\text{Volume of supernatant} (\mu\text{l})}$$

$$\text{NSB correction factor} = \frac{\text{Probe}_{\text{bound}}}{\text{Probe}_{\text{free}}}$$

The NSB correction factors were calculated for both salicylic acid and TPP<sup>+</sup> at each sample time and subtracted from the concentration ratios of the

respective probe. Initially, these correction factors were calculated from the plot of the concentration of the bound probe versus free probe for cells incubated with different concentrations of the probe. The NSB factor for each culture condition was then calculated from the slope of the line (Figures A1 and A2). We began calculating correction factors at each sample time after consultation with Dr. Thomas Montville. Culture age and medium composition affect the binding of the probes and thus it was necessary to calculate them at each sample time.

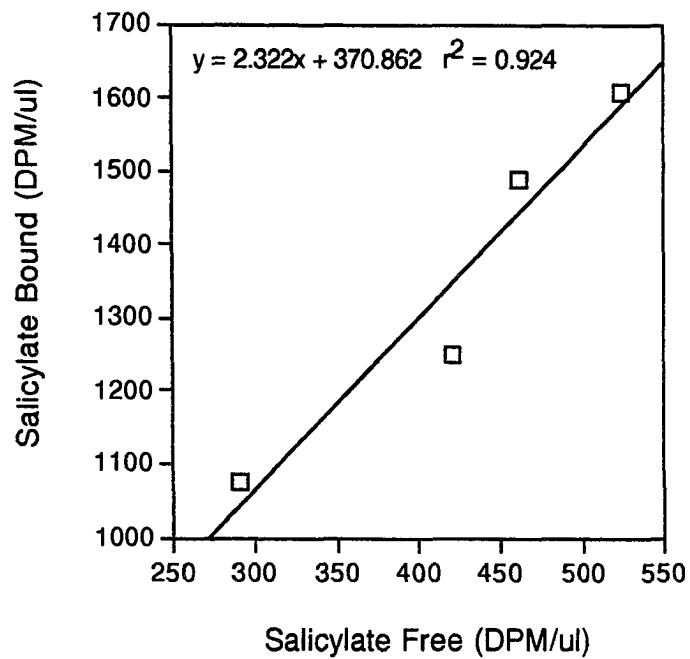
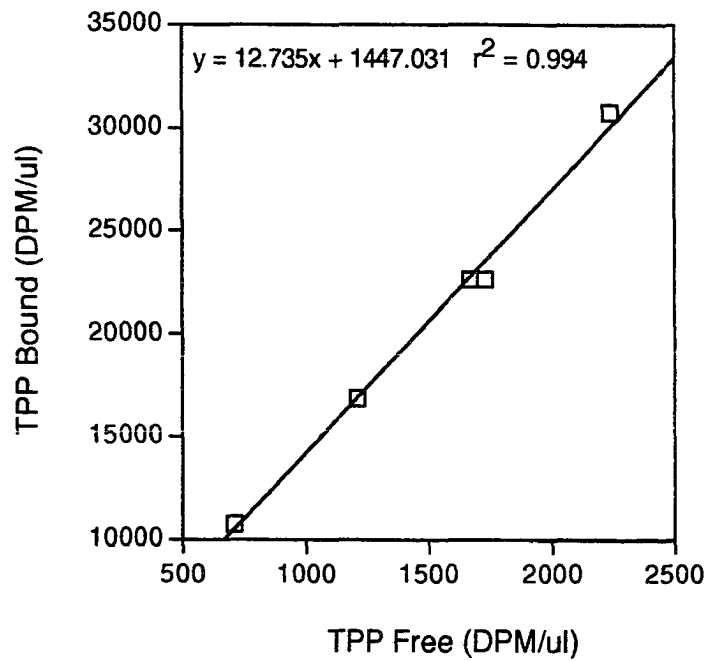


Figure A1: Bound versus free probe concentrations for a) TPP+ and b) salicylate for pH 7 cultures. NSB correction factors are given as the slope of the line.

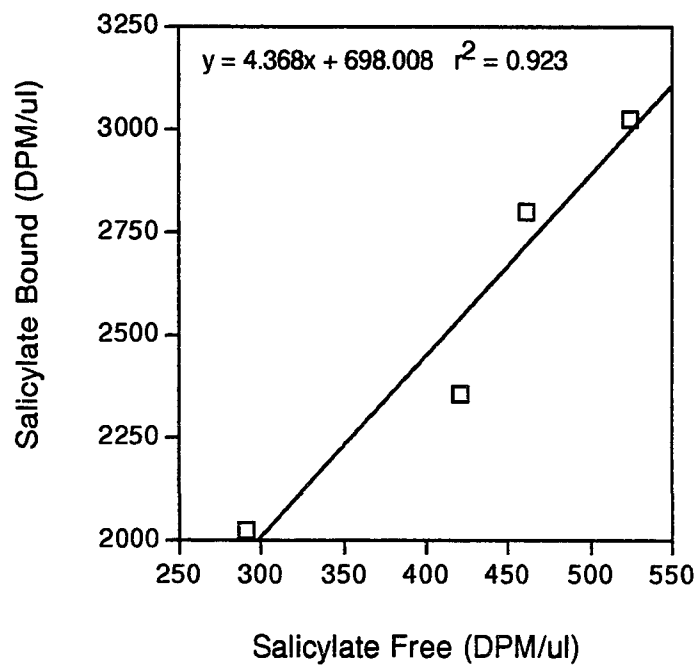
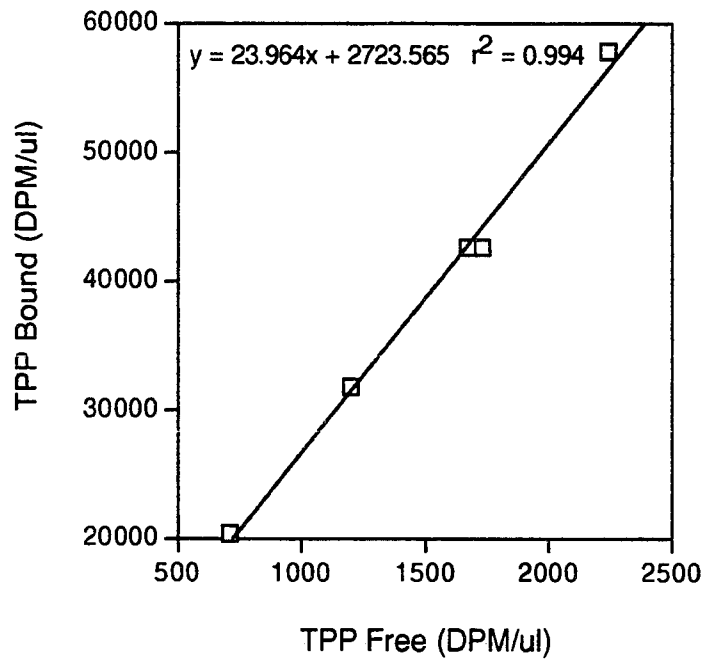


Figure A2: Bound versus free probe concentrations for a) TPP+ and b) salicylate for pH 5 cultures. NSB correction factors are given as the slope of the line.

### $\Delta\Psi$ Calculations

The  $\Delta\Psi$  was calculated based on the distribution of [ $^3\text{H}$ ] TPP $^+$  (2.25  $\mu\text{M}$ , 0.5  $\mu\text{Ci}$ ). A stock solution (450  $\mu\text{M}$ , 0.1  $\mu\text{Ci}/\mu\text{l}$ ) was prepared from 0.1 ml of [ $^3\text{H}$ ] TPP $^+$  (1  $\mu\text{Ci}/\mu\text{l}$ ) and 0.9 ml of 500  $\mu\text{M}$  "cold" TPP $^+$ . The  $\Delta\Psi$  was calculated from:

$$\text{TPP}^+_{\text{out}} (\text{DPM} / \mu\text{l}) = \frac{\text{DPM supernatant} - \text{DPM Bkgd}}{\text{Volume of supernatant} (\mu\text{l})}$$

$$\text{TPP}^+_{\text{in}} (\text{DPM} / \mu\text{l}) = \frac{\text{DPM pellet} - \text{DPM Bkgd}}{[\text{internal volume} (\mu\text{l}/\text{mg})][\text{pellet weight} (\text{mg})]}$$

$$\text{TPP}^+_{\text{corrected}} = \frac{\text{TPP}^+_{\text{in}}}{\text{TPP}^+_{\text{out}}} - \text{NSB correction factor}$$

$$\Delta\Psi = -Z \log(\text{TPP}^+_{\text{corrected}})$$

Where  $z = 2.303 RT/nF$ ;  $R$  = gas constant (8.31 J/mol K);  $T$  = absolute temperature (K);  $n$  = charge of transported species = 1;  $F$  = Faraday's constant (96.519 J/mol mV).  $z$  is 59 mV at 25°C

### **$\Delta$ pH Calculations**

The  $\Delta$ pH was calculated from the distribution of [ $^{14}$ C] salicylic acid (2.5  $\mu$ M, 0.125  $\mu$ Ci). The stock solution was prepared with 0.24 ml of [ $^{14}$ C] salicylic acid (1800  $\mu$ M, 100  $\mu$ Ci) and 0.76 ml of 100  $\mu$ M “cold” salicylic acid to give a final concentration of 500  $\mu$ M and 0.025  $\mu$ Ci/ $\mu$ l. Salicylic acid was chosen because of its low  $pK_a$  (2.97), which allowed 99% dissociation of the probe in the extracellular and intracellular space even at pH 5. The  $\Delta$ pH was calculated from:

$$\text{Salicylic acid}_{\text{out}} (\text{DPM} / \mu\text{l}) = \frac{\text{DPM supernatant} - \text{DPM Bkgd}}{\text{Volume of supernatant} (\mu\text{l})}$$

$$\text{Salicylic acid}_{\text{in}} (\text{DPM}/\mu\text{l}) = \frac{\text{DPM pellet} - \text{DPM Bkgd}}{[\text{internal volume} (\mu\text{l}/\text{mg})][\text{pellet weight} (\text{mg})]}$$

$$\text{Salicylic acid corrected} = \frac{\text{salicylic acid}_{\text{in}}}{\text{salicylic acid}_{\text{out}}} - \text{NSB correction factor}$$

$$\Delta\text{pH} = \log(\text{salicylic acid corrected})$$

When the pH of the medium was  $< pK_a + 1$  (i.e. 3.97) the following formula was used:

$$\Delta\text{pH} = \log\left\{(\text{salicylic acid corrected})\left(10^{pK_a - \text{pH}_{\text{out}}} + 1\right) - 10^{pK_a - \text{pH}_{\text{out}}}\right\}$$



$$pH_{in} = pH_{out} + \Delta pH$$

The total  $\Delta p$  was calculated as:

$$\Delta p = \Delta \Psi - Z\Delta pH$$

The  $\Delta \Psi$  and  $\Delta pH$  were considered to be collapsed when the concentration ratio minus the NSB correction factor was  $< 1$ , which would result in a negative value after taking the logarithm. In these cases the  $\Delta \Psi$  was not reported as positive;  $TPP^+$  would not accumulate in cells with a positive interior and thus the values were reported as zero. Similarly, the  $\Delta pH$  was reported as zero in these cases because salicylic acid would not accumulate in cells that were more acidic than the external medium.

## **APPENDIX B: EXTENDED FERMENTATION OF *P. THOENII* P 127 AT PH 7**

During extended incubation (480 to 1500 h) of *P. thoenii* P127 in batch culture controlled at pH 7, the culture experienced an increase in the proton motive force, viable cell counts, and acetic acid concentrations. The purity of the culture was verified by microscopic examination and plating onto sodium lactate agar and Brain Heart Infusion agar (Difco), which would allow a variety of fastidious organisms to grow. The plates were incubated aerobically and anaerobically at 32°C for five to seven days. The culture was also plated onto Sulfite Polymixin Sulfadiazine agar (Difco) and incubated anaerobically at 37°C for 48 h to check for the presence of clostridia.

These results may suggest that the culture had undergone some metabolic change which may have induced the synthesis of new enzymes. To get an overall view of cellular proteins, the intracellular and membrane-bound proteins of the culture at 1516 h and a control culture at 48 h were separated by SDS-PAGE.

The cells were harvested by centrifugation (9,000 x *g*, 15 min), washed in 10 mM MES (Fisher), and resuspended in a small amount of 10 mM MES to form a thick cell paste. The cell suspension was disrupted by passing through a chilled French pressure cell maintained at 18,000 lbs/in<sup>2</sup>. Three passes were necessary to obtain adequate cell disruption. Cell debris and remaining intact cells were removed by centrifugation at 9,000 x *g* for 15 min. Aliquots (3-5 µl) of supernatant were subjected to SDS-PAGE. Electrophoresis was performed using 7.5% Tris-

HCl polyacrylamide gel (Mini-PROTEAN II Ready Gels; BioRad Laboratories, Hercules, CA) and 10-20% gradient Tris-HCl polyacrylamide gel (Jule Inc. Biotechnologies, New Haven, CT) with Tris-Glycine-SDS running buffer at constant voltage (200 V) for 30 min. Gels were stained with coomassie blue stain according to the manufacturer's instructions.

There were no significant differences in banding patterns of the larger molecular weight proteins on the 7.5% polyacrylamide gel (data not shown). However, there appeared to be an additional protein band on the 10-20% gradient polyacrylamide gel at approximately 25 kDa in the 1516 h culture (Figure B1). A better approach to detecting differences in protein content would be to conduct a two-dimensional-PAGE, the first dimension using isoelectric focusing and the second dimension using SDS-PAGE.

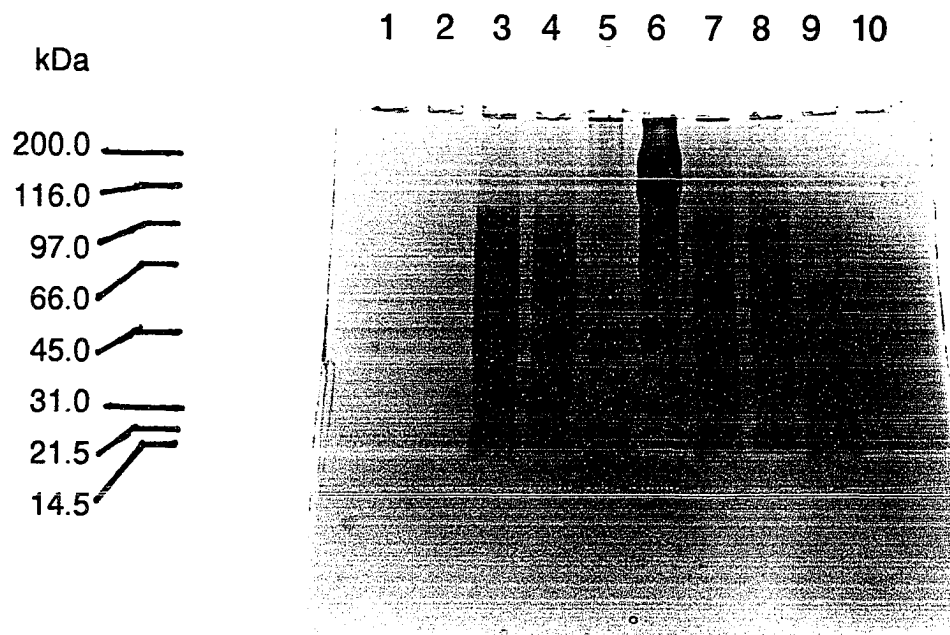
The cells may have synthesized enzymes for the metabolism of the remaining amino acids in the medium. Propionibacteria have been found to utilize several amino acids, especially aspartate, alanine, serine, and glycine (3). The metabolism of aspartate yields succinate, acetate, carbon dioxide, and ammonia. In addition, strains of propionibacteria have been found to produce polysaccharides (1). The formation of polysaccharides also increased the formation of acetic acid and carbon dioxide without any associated propionic acid production.

This additional band might also correspond to a protein synthesized during stationary phase. A variety of proteins have been found to be synthesized in *E. coli* during entry into stationary phase (2). These proteins were involved in maintaining viability during prolonged starvation.

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**Figure B1: SDS-PAGE analysis of P127 after 1516 h and 36 h (control) of incubation at pH 7 on a 10-20% gradient polyacrylamide gel. Lanes 1, 2, and 10: empty; Lanes 3 and 7: 1516 h cell lysate; Lanes 4 and 8: 36 h cell lysate; Lane 5: low molecular weight standard (Sigma); Lane 6: broad molecular weight standard (BioRad); Lane 9: colored low molecular weight standard (Sigma).**



## **APPENDIX C: PIGMENT LOSS**

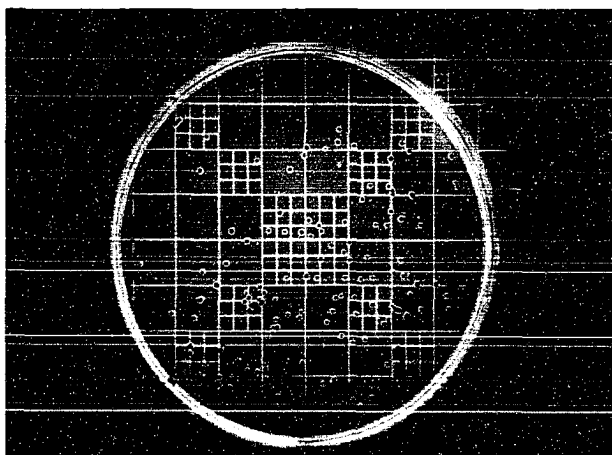
During the acid adaptation experiment, we noted that upon exposure to pH 3.5 and 3.0 a significant percent of colonies lost their typical red-orange pigment when plated onto nonselective medium (Figure C1). The percentage of unpigmented colonies increased with time of exposure to low pH. However, pigment loss was not permanent; when unpigmented cultures were subcultured in neutral laboratory medium for 36 to 48 h and plated onto nonselective medium, approximately half of the resulting colonies were pigmented.

Strain P127 also lost pigmentation upon exposure to streptomycin, valinomycin, and chloramphenicol, as well as during heat shock and when grown on extremely overcrowded plates. The percentage of pigment loss was greater for cultures that had experienced a significant cidal effect from the stress (Figure C2). The combination of low pH and exposure to chloramphenicol presented two "hurdles" for the culture to overcome. Survivors of this combination showed the highest percentage loss of pigment. This suggests that pigment loss is associated with stress, e.g. low pH, presence of antibiotics, heat shock, or competition for nutrients.

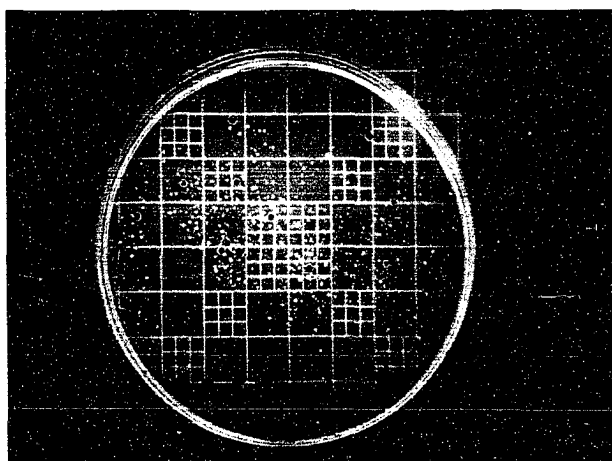
Figure C1: Photographs of plates of P127 typically seen upon exposure to stress.  
A) typical healthy colonies, B) unadapted (pH 7) culture after exposure to pH 3.5 for 60 min and C) adapted (pH 5.5) culture after exposure to pH 3.5 and chloramphenicol for 60 min.



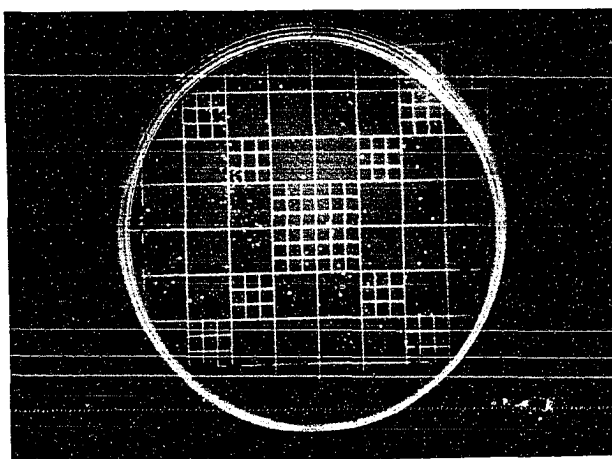
A



B



C



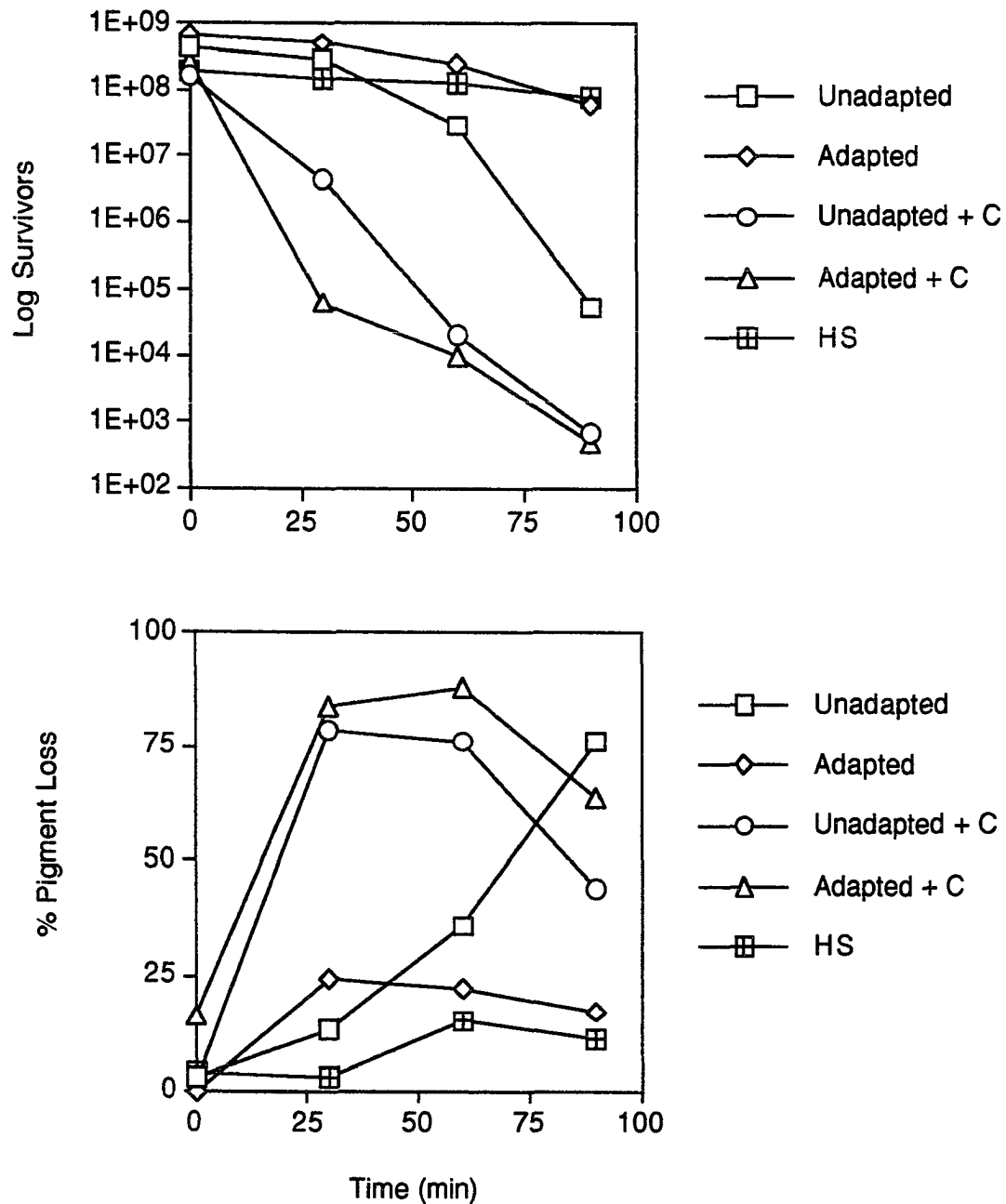


Figure C2: Comparison of total survivors and percent of pigment loss in cultures of P127 exposed to various stresses. Unadapted = cultures grown at pH 7 and then shifted to pH 3.5, Adapted = cultures exposed to pH 5.5 for one doubling prior to shifting to pH 3.5, C = chloramphenicol (100  $\mu$ g/ml), HS = heat shock at 55°C.

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